



## Review

## Biochemical consequences of mutations causing the GM2 gangliosidoses

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## Abstract

The hydrolysis of GM2-ganglioside is unusual in its requirements for the correct synthesis, processing, and ultimate combination of three gene products. Whereas two of these proteins are the  $\alpha$ - (*HEXA* gene) and  $\beta$ - (*HEXB*) subunits of  $\beta$ -hexosaminidase A, the third is a small glycolipid transport protein, the GM2 activator protein (*GM2A*), which acts as a substrate specific co-factor for the enzyme. A deficiency of any one of these proteins leads to storage of the ganglioside, primarily in the lysosomes of neuronal cells, and one of the three forms of GM2-gangliosidosis, Tay–Sachs disease, Sandhoff disease or the AB-variant form. Studies of the biochemical impact of naturally occurring mutations associated with the GM2 gangliosidoses on mRNA splicing and stability, and on the intracellular transport and stability of the affected protein have provided some general insights into these complex cellular mechanisms. However, such studies have revealed little in the way of structure–function information on the proteins. It appears that the detrimental effect of most mutations is not specifically on functional elements of the protein, but rather on the proteins' overall folding and/or intracellular transport. The few exceptions to this generalization are missense mutations at two codons in *HEXA*, causing the unique biochemical phenotype known as the B1-variant, and one codon in both the *HEXB* and *GM2A* genes. Biochemical characterization of these mutations has led to the localization of functional residues and/or domains within each of the encoded proteins. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Lysosomal storage disease; Tay–Sachs disease; Sandhoff disease; AB-variant form;  $\beta$ -Hexosaminidase; GM2 activator protein

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Abbreviations: Hex, human  $\beta$ -hexosaminidase; Activator, the human GM2 activator protein; GM1, GM2, GM3, GA2, see Table 3; GSL, glycosphingolipid; ER, endoplasmic reticulum; GlcNAc,  $\beta$ -D-N-acetylglucosamine; GalNAc,  $\beta$ -D-N-acetylgalactosamine; MUG, 4-methylumbelliferyl 2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside; 4-MUGS, 2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside-6-sulfate; Sp-Hex, hexosaminidase from *Streptomyces plicatus*; TDS, Tay–Sachs disease; SD, Sandhoff disease

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## 1. Introduction

Lysosomes are acidified cytoplasmic organelles which contain an array of degradative enzymes specialized for the orderly destruction of cellular components. The importance of the lysosomal system is emphasized by the fact that over 35 recessively inherited lysosomal storage diseases have been described in humans, most of which are the result of a primary deficiency of a single lysosomal enzyme. These diseases are characterized by the extensive vacuolization of cells resulting from the accumulation of the deficient enzyme's substrate(s). Among the hydrolytic enzymes in the lysosome are a group of exo-glycosidases which catalyze the sequential removal of terminal, non-reducing sugar units from the oligosaccharide component of a variety of large molecules. This review will deal primarily with the  $\beta$ -hexosaminidase ( $\beta$ -*N*-acetylhexosaminidase) isozymes and the GM2 activator protein (Activator). The former are lysosomal isozymes that cleave the glycosidic linkages of the non-reducing, terminal  $\beta$ -D-*N*-acetylglucosamine (GlcNAc) or  $\beta$ -D-*N*-acetylgalactosamine (GalNAc) residues on glycolipids, glycoproteins, and glycosaminoglycans. The Activator is a substrate specific (GM2 ganglioside) co-factor for one of the hexosaminidase isozymes, hexosaminidase A.

The presence of an *N*-acetyl- $\beta$ -D-glucosaminidase (NAG) was first reported in 1936 [1]. It was later found that unlike bacterial NAGs, the mammalian enzyme has an acidic pH optimum, is found predominantly in lysosomes, and cleaves the terminal non-reducing,  $\beta$ -1,4-linked, glycosidic bonds of both amino sugars, i.e. GlcNAc and GalNAc. Thus, the appropriate name of the enzyme is  $\beta$ -hexosaminidase or simply Hex (EC 3.2.1.52). Interestingly, in some segments of the literature, usually dealing with human Hex in body fluids, Hex is still incorrectly referred to as NAG, e.g. [2], which also has an alternative EC number, EC 3.2.30.

### 1.1. Classical Tay–Sachs disease, i.e. the acute infantile form

The early interest in Hex, which has led to its becoming the primary model for lysosomal glycosidases, was derived from its association with Tay–Sachs disease. The 'classical' infantile form (see below) of Tay–Sachs disease (TSD) has a carrier frequency of about 0.032 in the Ashkenazi Jewish population (0.0039 in the general population) and is one of the oldest known lysosomal storage diseases. It was first described by Dr. Warren Tay, a British ophthalmologist, in 1881 (Table 1) and results from the intralysosomal accumulation of GM2 ganglioside

Table 1  
History of the GM2 gangliosidoses

Year	Event	Reference
1881	Warren Tay: initial description of clinical characteristics, cherry red spot	[157]
1896	Bernard Sachs: 'Amaurotic family idiocy' prevalent in the Jews	[158]
1939	Identification of a novel class of compounds (gangliosides) stored in amaurotic idiocy	[159]
1962	GM2 ganglioside identified as the compound stored in TSD	[160]
1968	Demonstration of two major Hex isozymes in normal human tissue (Hex A and Hex B)	[5]
1969	Hex A, but not Hex B is deficient in Jewish TSD	[6]
1969	Isozyme patterns in non-Jewish TSD patients: (a) deficiency of both Hex A and B (SD); (b) no Hex deficiency (AB-variant or B1-variant)	[7]
1973	Immunological basis for the subunit compositions of Hex A ( $\alpha\beta$ ) and B ( $\beta\beta$ ): deficiency of the $\alpha$ results in TSD, deficiency of $\beta$ results in SD	[161]
1979	Purification and characterization of the GM2 Activator protein: its deficiency results in the AB-variant form	[162]
1984–1985	Isolation of cDNA clones encoding the $\alpha$ - and $\beta$ -subunits	[69,163]
1986	Identification of partial <i>HEXA</i> and <i>HEXB</i> gene deletions resulting in infantile TSD and SD (Table 4, row 1; Table 5, row 3)	[18,76]
1988	Identification of two major <i>HEXA</i> mutations responsible for TSD in Ashkenazi Jews (Table 4, rows 74 and 77)	[164–166]
1989–1992	Isolation of a cDNA clone encoding the GM2 activator	[74,75,167]
1991–1992	Association of a <i>GM2A</i> mutation with the AB-variant form (Table 6, row 3)	[154,155]

(Table 2), primarily in neuronal cells (reviewed in [3]).

Clinically classical infantile Tay–Sachs patients (or patients with other infantile forms of GM2 gangliosidosis, see below) show normal development until 3–6 months of age, at which time motor weakness, hypotonia, poor head control and decreasing attentiveness begin to cause parental concern. One of the earliest signs noted by parents is often an exaggerated startle reaction to sharp (not necessarily loud) sounds, characterized by a sudden extension of both arms and legs. By 10–12 months of age, any motor skills which had been achieved earlier, such as crawling and sitting, are lost. Over the next year, vision diminishes, although distinction between light and dark may persist, while direct or indirect ophthalmoscopy reveals macular pallor with contrasted prominence of the macular fovea centralis (the 'cherry red spot' which initially attracted Dr. Tay's attention). Enlargement of the head, also characteristic of this disorder, is the result of reactive cerebral gliosis, rather than true hydrocephaly with ventricular enlargement. By 18 months, neurologic signs of both upper and lower motor neuron deterioration become increasingly evident, with seizures becoming a common occurrence. Further deterioration in the second

year of life invariably leads to the disappearance of swallowing and gag reflexes, and eventually to a completely vegetative state. Bronchopneumonia associated with aspiration and or diminished capacity to cough, is frequently antecedent to death. Classical Tay–Sachs patients usually do not survive beyond 4 years of age [3,4]. This form of Tay–Sachs disease is found with greatest frequency in the French-Canadian (Table 4, row 1), Cajun (Table 4, row 74), and Ashkenazi (Table 4, rows 74 and 77) and Moroccan (Table 4, rows 25, 34 and 56) Jewish populations. Fortunately, however, the advent of international carrier detection programs has reduced its incidence in these groups by 90%.

### 1.2. The biochemical link between Hex activity and the GM2 gangliosidoses

Understanding the association between Hex activity and disease was a complex process for several reasons. Firstly, whereas the link between Tay–Sachs disease and a defect in Hex activity was suspected after the structure of the storage material, GM2 ganglioside, was determined (Tables 1–3), total Hex activity in samples from Jewish Tay–Sachs patients, as measured with artificial substrate, was nearly normal.

Table 2

Storage of GSL in the GM2 gangliosidoses

	GM2 <sup>a</sup>	GD2 <sup>a</sup>	GD1a <sup>a</sup>	GalNAc-GD1a <sup>a*</sup>	GA2 <sup>a</sup>	Gb4 <sup>b</sup>
Control	20	40	500	2	0	180
Tay–Sachs	10 000	80	400	30	1 000	400
Sandhoff	9 000	100	300	10	5 000	2 000
AB-variant	18 000	90	200	2	4 000	200

<sup>a</sup>Total brain GSL, nmol of ganglioside per g wet weight of tissue ([4] or \*[30]).<sup>b</sup>Total kidney globoside, nmol of globoside per g wet weight of tissue [4].

It was not until 1968 when Robinson and Stirling separated the total Hex activity from human spleen into two pI forms (an acidic, Hex A, and a basic, Hex B, form) [5], and Okada and O'Brien found that Hex A was missing in their Jewish patients that it was recognized that total Hex activity was derived from at least two gene products [6], i.e. Hex A and Hex B were true Hex isozymes (see below and Table 1). In Jewish Tay–Sachs patient samples Hex B levels increase sufficiently to mask the lack of Hex A.

Secondly, in contrast to the straightforward Hex A-deficiency found in Jewish patients, Sandhoff analyzed samples from both Jewish and non-Jewish 'Tay–Sachs' patients and found that some of the latter were missing both Hex A and Hex B or even more confusingly, appeared to have normal levels of both isozyme [7,8]. These results were not understood until the structure of the Hex isozymes, their substrate specificities, and the role of the Activator protein had been determined.

Thirdly, solid structural data on the isozymes were difficult to obtain. We now know that, whereas Hex B is a homodimer of  $\beta$ -subunits, Hex A is a heterodimer consisting of an  $\alpha$ - and a  $\beta$ -subunit. Two evolutionarily related genes encoded these subunits,  $\alpha$  by

the *HEXA* gene (15q23–q24, [9]) and  $\beta$  by the *HEXB* gene (5q13 [10]). Thus, Jewish Tay–Sachs disease results from *HEXA* mutations which only affect Hex A formation, and patients lacking both isozymes have another form of GM2 gangliosidosis, Sandhoff disease, caused by *HEXB* mutations which affects the common  $\beta$ -subunit (Table 5). Confusion over the structures of each isozyme, including the number of subunits in each and their relationship to each other, as well as the number of polypeptides contained within the subunits existed for sometime. The confusion arose because in vivo Hex is a rare protein requiring 6000–10 000 fold purification from human tissues, and its intra- and extra-cellular isozymes are heterogeneous with respect to both their subunits' polypeptide chain structures and carbohydrate contents. Thus each isozyme has multiple pI and apparent molecular weight ( $M_r$ ) forms (particularly when viewed by SDS-PAGE) [11–13]. This heterogeneity results from the initial gene products, the prepro- $\alpha$ - and  $\beta$ -polypeptides, undergoing extensive post-translational modifications starting in the endoplasmic reticulum (ER) (where they are synthesized) and Golgi, followed by final proteolytic and glycosidic processing in the lysosome to produce the mature

Table 3

GLS structures

Glycolipid	Oligosaccharide structure
GalNAc-GD1a	GalNAc $\beta$ (1–4)-Gal-[ $\alpha$ (2–3)NeuAc-] $\beta$ (1–3)GalNAc $\beta$ (1–4)Gal [ $\alpha$ (2–3)-NeuAc] $\beta$ (1–4)Glc-R <sup>a</sup>
GD1a	Gal-[ $\alpha$ (2–3)NeuAc-] $\beta$ (1–3)GalNAc $\beta$ (1–4)Gal [ $\alpha$ (2–3)-NeuAc] $\beta$ (1–4)Glc-R
GM1	Gal $\beta$ (1–3)GalNAc $\beta$ (1–4)Gal-[ $\alpha$ (2–3)NeuAc-] $\beta$ (1–4)Glc-R
GD2	Gal[ $\alpha$ (2–3)NeuAc- $\alpha$ (2–8)NeuAc-] $\beta$ (1–4)Glc-R
GM2	GalNAc $\beta$ (1–4)-Gal[ $\alpha$ (2–3)NeuAc-] $\beta$ (1–4)Glc-R
GM3	Gal[ $\alpha$ (2–3)NeuAc-] $\beta$ (1–4)Glc-R
GA2	GalNAc $\beta$ (1–4)Gal $\beta$ (1–4)Glc-R
Gb4	GalNAc $\beta$ (1–4)Gal $\alpha$ (1–4)Gal $\beta$ (1–4)Glc-R

<sup>a</sup>R = ceramide.

Table 4  
HEXA gene mutations associated with Tay–Sachs disease

Mutation	Location	Result	Biochemical phenotype	Clinical phenotype	Heritage	Ref.
1. $\Delta 7.6$ kb	5' to 1-IVS	No mRNA	Abnormal Southern blot, no mRNA	Acute	82% of French-Canadian TSD	[77,78,168]
2. A1 $\rightarrow$ T	1-exon	Met <sup>1</sup> $\rightarrow$ Leu	Protein initiation site	Chronic (second allele = Gly <sup>269</sup> Ser)		[169]
3. A1 $\rightarrow$ G	1-exon	Met <sup>1</sup> $\rightarrow$ Val	Protein initiation site	Acute	American Black	[170]
4. T2 $\rightarrow$ C	1-exon	Met <sup>1</sup> $\rightarrow$ Thr	Protein initiation site	Severe subacute (second allele = Pro <sup>25</sup> $\rightarrow$ Ser)	Non-Jewish (UK)	[171]
5. C73 $\rightarrow$ T	1-exon	Pro <sup>25</sup> $\rightarrow$ Ser	Hex A < 2.5% <sup>a</sup> , similar to Gly <sup>269</sup> $\rightarrow$ Ser mutation, soluble precursor no detectable mature form, transport mutant	Severe subacute (second allele = Met <sup>1</sup> $\rightarrow$ Thr)	Non-Jewish (UK)	[171]
6. G78 $\rightarrow$ A	1-exon	Trp <sup>26</sup> $\rightarrow$ Stop		Acute	UK, Israeli, Arab	[70,172]
7. T116 $\rightarrow$ G	1-exon	Leu <sup>39</sup> $\rightarrow$ Arg	Low level of pro- $\alpha$ no mature	Acute (second allele = exon 11 insertion)	Non-Jewish	[173]
8. C316 $\rightarrow$ T	2-exon	Gln <sup>106</sup> $\rightarrow$ Stop		Acute	Various	[174]
9. g $\rightarrow$ c	2-IVS+1	Abnormal splicing		Acute	European	[70]
10. g $\rightarrow$ a	2-IVS+1	Abnormal splicing	Low level of mRNA lacking exon 2	Acute	French	[175]
11. C379 $\rightarrow$ T	3-exon	Leu <sup>127</sup> $\rightarrow$ Phe		Acute	Various	[174]
12. T380 $\rightarrow$ G	3-exon	Leu <sup>127</sup> $\rightarrow$ Arg	50% mRNA	Acute (second = ?)	Italian	[173]
13. C409 $\rightarrow$ T	3-exon	Arg <sup>137</sup> $\rightarrow$ Stop	mRNA undetectable (second allele+TATC1278–1281)	Acute	French, Irish	[170,175]
14. g $\rightarrow$ t	3-IVS+1	Abnormal splicing	Skips exon 3	Acute	Japanese	[176]
15. $\Delta$ TT423–4	4-exon	$\Delta$ 142	No mRNA by Northern blot	Acute (homozygous)	French	[173]
16. $\Delta$ G436	4-exon	Frame shift (exon 6–Stop)		Acute (homozygous)	US, Black	[170]
17. +t	4-IVS+3t	skips exon 4 early Stop, 17 bp downstream	No normal mRNA severely reduced misspliced mRNA	Acute (homozygous)	Israeli-Bedouin	[177]
18. g $\rightarrow$ a	4-IVS+5	Abnormal splicing	3% mRNA lacking exon 4	Acute (second allele = +TATC1278–1281)	French	[175]
19. g $\rightarrow$ t	4-IVS–1	Abnormal splicing		Acute (homozygous)	US, Black	[178]
20. $\Delta$ TG477–8	5-exon	Frame shift	Stop = +2 codons	Acute	UK	[70]
21. C496 $\rightarrow$ G	5-exon	Arg <sup>166</sup> $\rightarrow$ Gly		Second allele = $\Delta$ C498	Syrian	[179]
22. $\Delta$ C496	5-exon	Frame shift	5% mRNA	Subacute (second allele = Ser <sup>297</sup> Pro)	Israeli Druze	[180]
23. $\Delta$ C498	5-exon	Frame shift, Stop 96 bp downstream	Stop = +32 codons	Second allele = Arg <sup>166</sup> Gly	Syrian	[179]
24. C508 $\rightarrow$ T	5-exon	Arg <sup>170</sup> $\rightarrow$ Trp		Acute (second = Gly <sup>454</sup> Ser)	Italian, French-Canadian	[70,148,173]

Table 4 (continued)  
*HEXA* gene mutations associated with Tay–Sachs disease

Mutation	Location	Result	Biochemical phenotype	Clinical phenotype	Heritage	Ref.
25. G509 → A	5-exon	Arg <sup>170</sup> → Gln (CpG)+abnormal splicing	mRNA splicing cryptic site –51 bp at 5'-end of exon. Patient cells; protein of reduced size. COS expression of missense as Hex S ( $\alpha^*\alpha^*$ ) formed in reduced amounts and is unstable	Acute	37% of TSD in Moroccan Jews; Japanese, Scottish	[173,181,182]
26. C532 → T	5-exon	Arg <sup>178</sup> → Cys (CpG)	B1-like catalytic defect; Hex S expression = inactive (likely some additional transport problems)	Acute	Czech	[141]
27. G533 → A	5-exon	Arg <sup>178</sup> → His (CpG)	B1-classic: normal mRNA and Hex A ( $\alpha^*\beta$ ) levels with MUG, but inactive with MUGS. COS expression = no Hex S ( $\alpha^*\alpha^*$ ) activity; Hex B (Arg <sup>211</sup> → His or Lys) analogs normal levels of mature $\beta^*\beta^*$ -CRM no activity with MUG	Subacute in homozygotes (severe subacute in compound with null allele)	Common in Portuguese, Diverse	[61,138–141,145]
28. G533 → T	5-exon	Arg <sup>178</sup> → Leu	B1-like, 19% serum Hex A (MUG) (likely some additional transport problems)	Acute (second allele = Trp <sup>26</sup> → Stop)	UK	[70]
29. T538 → C	5-exon	Tyr <sup>180</sup> → His	Protein unstable at physiological temperatures	Chronic	Ashkenazi Jewish	[117]
30. C540 → G	5-exon	Tyr <sup>180</sup> → Stop	Decreased mRNA	Acute	2% of TSD in Moroccan Jews	[182]
31. 547insA	5-exon	Frameshift	Stop = +2 codons, no detectable $\alpha$ -CRM	Acute (homozygous)	Chinese	[183]
32. G570 → A	5-exon–1	Silent mutation in last bp (Leu) of exon, CTG affects splicing	7% of mRNA lacking exon 5, 3% normal mRNA, 2.5% Hex A <sup>a</sup>	Severe subacute (homozygous)	Tunisian	[86,184]
33. g → a	5-IVS+1	Abnormal splicing		Acute	Turkish	[184]
34. a → g	5-IVS–2	Abnormal splicing			12% of TSD in Moroccan Jews	[185]
35. g → t	5-IVS–1	Abnormal splicing	Inframe deletion of exon 6, normal levels of shortened mRNA, protein is trapped in the ER, transport mutation	Acute (homozygous)	80% of TSD in Japanese	[96]*
36. (a) G574 → C, (b) G598 → A	6-exon	(a) Val <sup>192</sup> Leu is detrimental;(b) Val <sup>200</sup> Met (neutral)	Previously reported as a B1. Re-examined in transfected CHO cells and patient fibroblasts, only pro- $\alpha$ detected by Western blot, i.e. transport mutation	Acute second allele 9-IVS+1	European	[136,186–188]

Table 4 (continued)  
*HEXA* gene mutations associated with Tay-Sachs disease

Mutation	Location	Result	Biochemical phenotype	Clinical phenotype	Heritage	Ref.
37. A590 → C	6-exon	Lys <sup>197</sup> → Thr	Normal mRNA	Chronic (second allele = Arg <sup>499</sup> His)	Dutch	[173]
38. A611 → G	6-exon	His <sup>204</sup> → Arg		Acute (second allele = exon 11 insertion)	German	[173]
39. C629 → T	6-exon	Ser <sup>210</sup> → Phe	Suggested catalytic defect (unlikely), Normal mRNA	Acute	Algerian N. African	[175,189]
40. T632 → C	6-exon	Phe <sup>211</sup> → Ser	Normal mRNA	Acute (second allele = Arg <sup>499</sup> Cys)	Italian	[173]
41. C677 → T	6-exon	Ser <sup>226</sup> → Phe			Azore	[174]
42. g → a	6-IVS+1	Abnormal splicing	Skips exon 6, 50% mRNA	Subacute (second?)	US	[173]
43. C739 → T	7-exon	Arg <sup>247</sup> → Trp	Reduced activity and $\alpha$ -CRM, Hex A is formed but unstable	<i>Asymptomatic</i> in compounds with null allele	Diverse, ~5% of enzyme-based carriers in non-Jews, < 3% in Jews	[118,126, 190–193]
44. C745 → T	7-exon	Arg <sup>249</sup> → Trp	Reduced activity and $\alpha$ -CRM, Hex A is formed but unstable	<i>Asymptomatic</i> in compounds with null allele	French Canadian	
[70,118,127,191]						
45. G748 → A	7-exon	Gly <sup>250</sup> → Ser	Reduced activity, but not found in any patients with TSD	pseudo-deficiency?	French Canadian	[193]
46. G749 → A	7-exon	Gly <sup>250</sup> → Asp (CpG)	Hex A = 2–3% <sup>a</sup> ; COS expression, no Hex S activity, Hex A activity and CRM intermediate (12%), transport mutation	Subacute	Lebanese	[194,195]
47. G755 → A	7-exon	Arg <sup>252</sup> → His		Chronic	Portuguese	[196]
48. G772 → C	7-exon	Asp <sup>258</sup> → His	B1-like, patient sample = 16% Hex A with MUG, < 1% with MUGS; CHO expression of Hex B analog, $K_m$ = 3 × higher plus transport problems	Severe subacute		[148–150]
49. g → c	7-IVS+1	Abnormal splicing	mRNA undetectable	Acute (homozygote)	Portuguese	[197]
50. g → a	7-IVS+1	Abnormal splicing	mRNA undetectable	Acute (second allele = $\Delta$ 7.6 kb)	French-Canadian	[70,198]
51. g → a	7-IVS–7	Abnormal splicing	New 3'-splice acceptor 5 bp upstream, lacking exon 8 mRNA = 6%, normal mRNA = 10%	Chronic (second allele = exon 11 4 bp insertion)	Canadian/ English	[199]
52. G805 → A	7-exon–1	Gly <sup>269</sup> → Ser plus abnormal splicing	Hex A = 4–6% <sup>a</sup> ; 50% mRNA due to splicing defect. COS expression, Hex S ( $\alpha^*\alpha^*$ ) inactive, Hex A ( $\alpha^*\beta$ ) = 40%, however it is unstable at 37°C	Chronic with null allele, homozygotes at mild end of spectrum	Ashkenazi 3%, 5% in non-Jews with TSD	[87,88,143] [102,170, 200,201]
53. G806 → A	8-exon	Gly <sup>269</sup> → Asp				[174]
54. T835 → C	8-exon	Ser <sup>279</sup> → Pro		Severe subacute, second allele $\Delta$ C496	Israeli-Druze	[180]

Table 4 (continued)  
*HEXA* gene mutations associated with Tay–Sachs disease

Mutation	Location	Result	Biochemical phenotype	Clinical phenotype	Heritage	Ref.
55. T902 → G	8-exon	Met <sup>301</sup> → Arg	Normal mRNA	Acute (second = Arg <sup>504</sup> → His)	Yugoslavian	[173]
56. ΔTTC910–912	8-exon	ΔPhe <sup>304</sup> or <sup>305</sup>	Transport mutation; COS expression Hex S inactive, pro-α CRM only	Acute	43% of TSD in Moroccan-Jew(Irish, French)	
[170,175,182,202]						
57. ΔCT927–28	8-exon	Frame shift	Stop = +12 codons, no mRNA	Severe subacute (with B1)	German Scandinavian	[148]
58. A941 → T	8-exon	Asp <sup>314</sup> → Val			Ashkenazi Jews, Irish-English	[174]
59. ΔGGA958–961	8-exon	ΔGly <sup>320</sup> or <sup>321</sup>		Severe subacute	Irish	[170]
60. a → g	8-IVS+3	Abnormal splicing	Loss of exon 8	Severe subacute (second allele = Arg <sup>178</sup> His (B1))		[203]
61. G987 → A	9-exon	Trp <sup>329</sup> → Stop		Acute (if second allele = B1, severe subacute)	English, German	[170]
62. A1003 → T	9-exon	Ile <sup>335</sup> → Phe		Affected prenatal	Non-Jewish	[204]
63. 1039del18	9-exon	del 6 aa?		Affected prenatal	Ashkenazi Jewish	[204]
64. g → a	9-IVS+1	Abnormal splicing	Low levels of mRNA with several cryptic splices; no normal mRNA	Acute	Major non-Jewish mutation; 8% of Cajun, 17% of W. Europeans	[205–209]
65. 1074-Δtctcc	9-IVS–8–12	Abnormal splicing	Low mRNA, most missing exon 10	Acute (second allele+TATC1278)	Polish	[70]
66. g → t	9-IVS–1	Abnormal splicing		Acute	Irish, French	[210]
67. C1168 → T	11-exon	Gln <sup>390</sup> → Stop				[174]
68. G1171 → A	11-exon	Val <sup>391</sup> → Met		Chronic (second allele = Arg <sup>178</sup> His (B1))	Greek	[211]
69. G1176 → A	11-exon	Trp <sup>392</sup> → Stop		Acute	Ashkenazi	[212]
70. C1177 → T	11-exon	Arg <sup>393</sup> → Stop	No mRNA	Acute (homozygous)	French, Turkish	[175,184]
71. ΔG1182 or 1183	11-exon	Frame shift	Stop = +3 codons	Acute (obligate carrier)	US	[204]
72. 1204 del 3	11-exon	Lys <sup>402</sup> del				[174]
73. G1260 → C	11-exon	Trp <sup>420</sup> → Cys	COS expression, no Hex S activity; likely transport mutation	Acute	German	[213]
74. +TATC1278	11-exon	Frameshift	Stop = +4 codons; absent mRNA in patients' cells, normal transcription rate; COS expression = significant levels of mRNA producing truncated, pro-α-CRM	Acute	20% of Non-Jews, 75% of Ashkenazi Jews, 92% of Cajuns; 2% of Moroccan Jews	[97,164, 170,185,205]
75. G1360 → A	12-exon	Gly <sup>454</sup> → Ser	Normal mRNA	Acute (second allele = Arg <sup>170</sup> Trp)	Italian	[173]
76. G1373 → A	12-exon	Cys <sup>458</sup> → Try	COS expression = no Hex S activity	Acute	Japanese	[176]



Table 4 (continued)  
*HEXA* gene mutations associated with Tay–Sachs disease

Mutation	Location	Result	Biochemical phenotype	Clinical phenotype	Heritage	Ref.
77. c→g	12-IVS+1	Abnormal splicing	No normal mRNA, trace of abnormal mRNA retaining intron 12 or excluding exon 12	Acute	15% of TSD in Ashkenazi, 2% in Moroccan Jews	
[165,166,185,214] 78. G1422→C	13-exon	Trp <sup>474</sup> →Cys (1st nucleotide in exon)	Possibly some abnormal splicing. In patient sample 5% <sup>a</sup> Hex A; COS expression ( $\alpha^*\beta$ ) 12% Hex S+A, transport mutation	Subacute (second allele = 4 bp insertion)	Non-Jewish	[215]
79. G1444→A	13-exon	Glu <sup>482</sup> →Lys (CpG)	Patient sample, Hex A = < 1% <sup>a</sup> ; protein cannot exit ER, i.e. transport mutation	Acute	2% of TSD in Moroccan Jews; Chinese, Italian	
[183,185,216,217] 80. T1451→C	13-exon	Leu <sup>484</sup> →Pro	COS expression, no Hex S activity, likely transport mutation	Acute	Japanese	[176]
81. T1453→C	13-exon	Trp <sup>485</sup> →Arg	COS expression, no Hex S activity, likely transport mutation	Acute	Chinese	[183]
82. C1495→T	13-exon	Arg <sup>499</sup> →Cys		Acute (if second allele Gly <sup>269</sup> Ser (chronic))	Slavic, Irish, English, Polish	[170,218]
83. G1496→A	13-exon	Arg <sup>499</sup> →His (CpG)	Patient cells; decreased solubility of pro- $\alpha$ , most cannot exit ER, 3% Hex A <sup>a</sup> , transport mutation	Subacute	Scottish-Irish, Jewish	[70,218,219]
84. $\Delta$ C1510	13-exon	Frame shift, Stop+4 aa, loss of 22 aa	Normal levels of mRNA, truncated $\alpha$ that cannot exit ER and is degraded, transport mutation	Acute (homozygous)	Italian	[98,220]
85. C1510→T	13-exon	Arg <sup>504</sup> →Cys (CpG)	Inhibited subunit asso? COS expression, no Hex S dimers or activity; transport mutation	Acute (homozygous)	Diverse	
[173,175,176,221] 86. G1511→A	13-exon	Arg <sup>504</sup> →His (CpG)	Patient cells; $\alpha$ does not associate with $\beta$ , but is phos. and can be secreted with NH <sub>4</sub> Cl. COS expression Hex S fails to form, transport mutation	Subacute (homozygous)	Diverse	[176,219,222]
87. 1549 ins C	14-exon	frameshift		Acute	Ecuadorean	[174]

<sup>a</sup>Residual Hex A activity in patient cells (% normal) if known.

Table 5  
*HEXB* gene mutations associated with Sandhoff disease

Mutation	Location	Result	Biochemical phenotype	Clinical phenotype	Heritage	Ref.
1. 50 kb deletion	25 kb 5'-pro-moter-intron 6	No mRNA	No mRNA or $\beta$ -protein	Acute (second allele = Ser <sup>62</sup> Leu)		[83]
2. (50 kb) deletion: re-tested same as 16 kb below	1–5-exons+promoter	No mRNA	No mRNA or $\beta$ -protein, Same mutation as [18,79]	Acute	Diverse 11/28, 30% of SD alleles	[80,82]
3. 16 kb deletion	1–5-exons+promoter	No mRNA	No mRNA or $\beta$ protein	Acute	Diverse 8/30, 27% of SD alleles	[79]
4. $\Delta A_{76}$	1-exon	Frame shift		Acute	Cyprus Maronite	[223]
5. C <sub>185</sub> → T	1-exon	Ser <sup>62</sup> → Leu		Acute (second allele = $\Delta 50$ kb)		[83]
6. g → a	2-IVS+1	Abnormal splicing	Unstable mixture of abnormal mRNA, linked to Pst polymorph	Acute	Argentinean (common mutation)	[224–226]
7. A <sub>619</sub> → G	5-exon	Ile <sup>207</sup> → Val	Originally suggested impaired activator binding, but has now been shown to be a neutral polymorphism	Chronic, motor neuron disease (second allele = Tyr <sup>456</sup> → Ser)	Not reported	[83,227–229]
8. $\Delta CTTT_{782-5}$	7-exon	frameshift at Tyr <sup>260</sup>	Early Stop codon at 273, unstable mRNA	Acute	Argentinean	[224,226]
9. $\Delta G_{772}$	7-exon	Frameshift at Gly <sup>258</sup>	Early Stop at codon 274, unstable mRNA	Acute (second allele = $\Delta AG$ )	Not reported	[99,230]
10. C <sub>850</sub> → T	7-exon	Arg <sup>284</sup> → Stop	0.8% mRNA	Acute		[99]
11. G <sub>890</sub> → A	8-exon	Cys <sup>297</sup> → Tyr		Chronic (second allele = Pro <sup>417</sup> Leu)	Italian	[92]
12. C <sub>1252</sub> → T	11-exon+8	Abnormal splicing, Pro <sup>417</sup> → Leu is neutral	Unstable mRNA (P2 and P3) P1 = normal mRNA; 8% of normal mRNA levels of which 80% is P1, aa substitution is silent	(a) Subacute (homozygous); (b) very mild (second allele = $\Delta 16$ kb)	(a) Japanese; (b) French-Canadian; (c) Italian	(a) [71](b) [90](c) [92]
13. $\Delta AG_{1305-6}$	11-exon	Frameshift, at Arg/Val <sup>436</sup>	Early Stop at codon 454, 1.8% mRNA	Acute (second allele = $\Delta G$ )	Not reported	[99,230]
14. $\Delta T_{1344}$	11-exon	Frameshift	Early Stop at codon 451, 30% mRNA	Acute (second allele = $\Delta 16$ kb)		[99]
15. A <sub>1367</sub> → C	11-exon	Tyr <sup>456</sup> → Ser	No $\beta$ -protein, transport mutant (second disease allele reported to be Ile <sup>207</sup> → Val, but this substitution was found to be neutral)	Chronic, motor neuron disease (probably due to second unknown allele)	Not reported	[227–229]
16. g → a	12-IVS*–26	Abnormal splicing ('a' moves to –2 position)	Insertion of 8 aa, normal mRNA levels, > 97% Abnormal splicing, unstable elongated $\beta$ -chain, 3% Hex A <sup>a</sup> undetectable–Hex B	Subacute (second allele = $\Delta 16$ kb)	Caucasian, Japanese	[89,91,231]

Table 5 (continued)  
HEXB gene mutations associated with Sandhoff disease

Mutation	Location	Result	Biochemical phenotype	Clinical phenotype	Heritage	Ref.
17. C <sub>1510</sub> → T	13-exon	Pro <sup>504</sup> → Ser	Decreased transport out of the ER (~45%), specific 3-fold loss of activity towards GM2 as compared with artificial substrates, lower heat stability	Chronic (second allele = Δ16 kb)	French-Canadian	[29]
18. G <sub>1514</sub> → A	13-exon	Arg <sup>505</sup> → Gln	Heat labile Hex B	Chronic (second allele = Δ16 kb)		[124,232,233]
19. G1565 → A (G1601 → A)	13-exon	Cys <sup>522</sup> → Tyr (Cys <sup>534</sup> → Tyr)	(Corrected numbering given in brackets)	Acute	Japanese	[234]
20. +18 bp duplication	13-IVS* last 16 bp+first 2 bp exon 14	Abnormal splicing	Insertion of 6 aa, normal mRNA levels, unstable elongated β chain, 9–10% Hex A <sup>a</sup> undetectable Hex B	Asymptomatic	French, SD Paris	[44,89]
21. t → g	13-IVS+2	Abnormal splicing		Acute		[235]
22. G <sub>1627</sub> → A	14-exon	Ala <sup>543</sup> Thr	Heat labile Hex B	Chronic	Oriental, Jews, Arabs	[125,236]

<sup>a</sup>Residual activity presumably arises from some normally spliced mRNA.

forms (Figs. 1 and 2) (reviewed in [3,14,15]). The Hex in extracellular fluids originates from two sources. (a) The intracellular transport of Hex does not result in 100% of the protein being incorporated in the lysosome. Some of the Hex isozymes in their precursor forms escape this pathway and are secreted (Fig. 2). (b) Cell death releases mature forms of the Hex isozymes.

Fourthly, it was found that Hex A alone is not sufficient to hydrolyze GM2 ganglioside, *in vivo*, where the reaction requires a third gene product, the GM2 activator protein (Activator) encoded by the *GM2A* gene 5q 31.3–33.1 [16], as a ‘substrate specific co-factor’ (Fig. 3). This explains Sandhoff’s most confusing observation of a patient with apparently normal levels of both Hex isozymes.

In summary, mutations in any of three genes can lead to a blockage in GM2 degradation. Since gangliosides are synthesized primarily in neuronal cells, these are the cells most affected by the disease (Tables 2 and 3). When the storage of GM2 reaches some critical value these cells are destroyed, possibly through apoptosis [17], resulting in one of three diseases with similar clinical phenotypes, the GM2 gangliosidoses: (1) Tay–Sachs, α-defects (Table 4); (2) Sandhoff, β defects (Table 5); and (3) the AB-variant, Activator defects (Table 6).

For all the above reasons the literature dealing with Hex published in the 1970s and early 1980s contained numerous contradictory and/or incomplete models for the structures and relationships of the Hex isozymes (reviewed in [14,15]). The present era of solid structural data on the Hex isozymes (reviewed in [3]) has been ushered in by the isolation of cDNA clones encoding the two Hex subunits, as well as those encoding the Activator protein (Table 1).

## 2. The Hex isozymes

There are two major Hex isozymes in normal human tissue. Hex A (*pI*=4.8) is a heterodimer, αβ, whereas Hex B (*pI*=6.9) is a homodimer, ββ. A third, unstable isozyme, Hex S (*pI*=3.4), is composed of two α-subunits. Hex S accounts for the 1–6% of normal activity found in human cells that do not synthesize functional pro-β-polypeptides, *i.e.*



gene and it would be expected that the  $\alpha$ - and  $\beta$ -subunits have the same overall three-dimensional structure. This hypothesis is consistent with the ability to form dimers with similar substrate specificities from any combination of the two subunits (see below) [25].

Although only dimeric forms of Hex are active, the existence of the three Hex isozymes ( $\beta\beta$ ,  $\alpha\beta$ , and  $\alpha\alpha$ ) indicates that each subunit contains all the elements necessary to form one active site. The active sites associated with both the  $\alpha$ - and  $\beta$ -subunits hydrolyze many of the same neutral artificial substrates (common substrates), e.g. MUG (4-methylumbelliferyl 2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside) [25], and several natural ones (reviewed in [15]). However, only the presence of the active site associated with the  $\alpha$ -subunit results in the efficient hydrolysis of negatively charged substrates, e.g.  $\beta$ -linked *N*-acetylglucosamine 6-sulfate containing glycosaminoglycans [25–27] and artificial substrates [25,27], e.g. 4-MUGS (2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside-6-sulfate) [28], and, most importantly, GM2 ganglioside (Fig. 3). In the latter case the  $\beta$ -subunit also plays a role, presumably in substrate (Activator:GM2 ganglioside complex) binding [29], as only the  $\alpha$ -subunit in its heterodimeric Hex A isozyme is functional *in vivo* [30]. The  $K_m$  and  $V_{max}$  of Hex A and B for MUG are nearly identical ( $K_m = 0.7$ – $0.9$  mM and  $V_{max} = 10$ – $20$  mmol h<sup>-1</sup> mg<sup>-1</sup>) [25,31]. The  $K_m$  for MUGS is 0.2–0.3 mM for Hex A and about ten-

times this concentration for Hex B (it is difficult to calculate for Hex B because of the limited solubility of the substrate). This does not mean that Hex B cannot hydrolyze MUGS only that its rate of hydrolysis at normal assay concentrations is about 1.3% that of Hex A, i.e. MUG:MUGS = 4:1 for Hex A and 300:1 for Hex B [25].

### 3. Mechanism of GM2 hydrolysis

GM2 is an intermediate in both the synthesis (ER) and degradation (lysosome) of the higher brain gangliosides, e.g. GM1 (Table 3). In order to hydrolyze GM2, Hex A requires the small, monomeric, heat stable GM2 activator protein (Activator, Fig. 3). The Activator interacts with both the carbohydrate and lipid portion of the ganglioside, and it eliminates steric hindrance from the membrane by solubilizing a ganglioside molecule [30]. As well, Activator binding may interrupt a specific hydrogen bond between the acetamido-NH of the GalNAc residue and the carboxylic group of the NeuAc residue of GM2, making the GalNAc available for hydrolysis by Hex A [32]. The relative importance of these two functions is currently a matter of debate in the literature (reviewed in [33]). The Activator:GM2 complex then specifically interacts with Hex A with a  $K_m$  of  $\sim 0.2$   $\mu$ M [34]. However, its rate of MUGS:GM2 hydrolysis is  $\sim 800$ :1 [25]. Surprisingly, this indicates

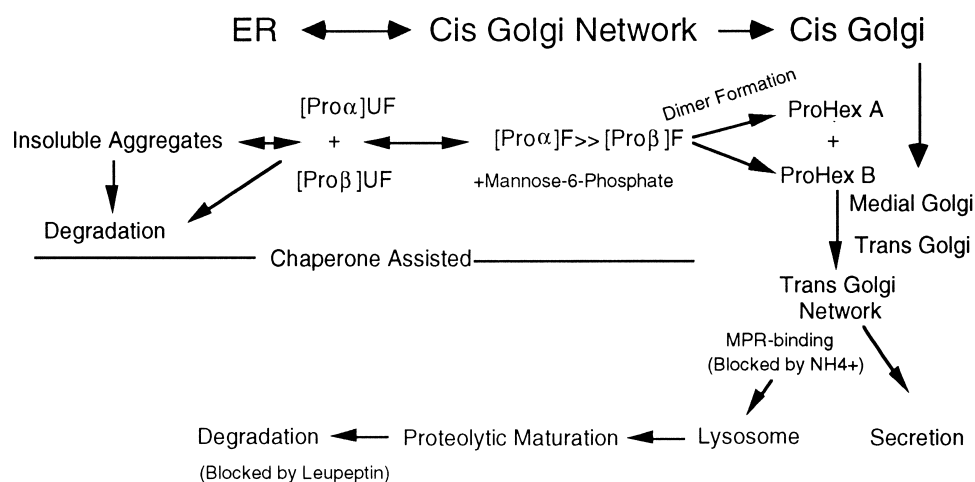


Fig. 2. Flow chart depicting the biosynthesis, intracellular transport and assembly of the Hex isozymes. [Pro $\alpha/\beta$ ]UF indicates the concentration of unfolded  $\alpha$ - or  $\beta$ -pro-polypeptides, whereas [Pro $\alpha/\beta$ ]F indicates the concentration of either folded subunit. Transport from the *cis* Golgi to the TGN is through bulk-flow with secretion being the cell's default pathway.

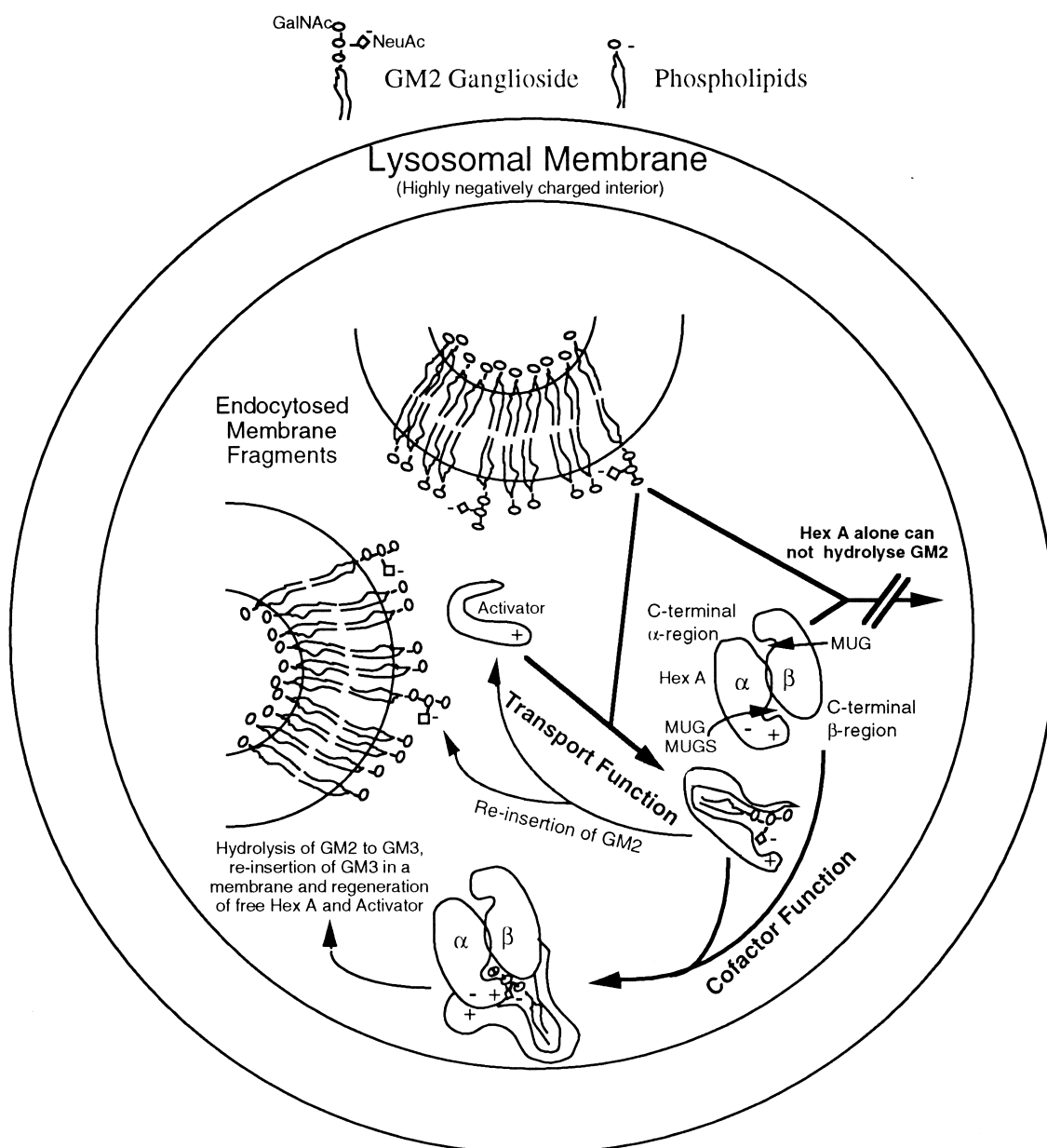


Fig. 3. Cartoon outlining the steps in the degradation of GM2 ganglioside by Hex A and the Activator (adapted from [4]).

that Hex A's second order rate constants, i.e. its catalytic efficiency, are similar for artificial and natural substrates, i.e.  $k_{\text{cat}}/K_m$  ( $800/0.25 \times 10^{-3}$  M (MUGS)  $\sim 1/0.2 \times 10^{-6}$  M (Activator:GM2)). In vivo substrate specificities and the need for the presence of the Activator can also be inferred from the compounds that are stored in the various GM2 gangliosidoses (Table 2).

In vitro assays can utilize detergents as a substitute for the Activator. Under these conditions Hex S, as

well as Hex A, but not Hex B, can efficiently hydrolyze GM2. Interestingly, Hex B can hydrolyze GA2, the neutral, asialo derivative of GM2 in the presence of detergent, but has little or no activity in the presence of activator alone. As well the Activator, even in the absence of GM2, has been reported to inhibit the hydrolysis of MUGS by both Hex A and Hex S (reviewed in [4,35]). These data indicate that the binding site for the complex, as well as the negatively charged substrate, is located in the  $\alpha$ -subunit, but

Table 6

*GM2A* gene mutations associated with the AB-variant form of GM2 gangliosidosis

Mutation	Location	Result	Biochemical phenotype	Clinical phenotype	Heritage	Ref.
1. ΔAAG262–264	3-exon	ΔLys <sup>88</sup>	Normal mRNA, no mature CRM, transport mutation; $8.4 \pm 0.4\%$ residual activity after bacterial expression	Acute (homozygous)	Saudi Arabian consanguinity	[237]
2. ΔA410	3-exon	33 new aa, loss of 24	Normal mRNA, no mature CRM, transport mutation; $3\% \pm 0.2$ residual activity after bacterial expression	Acute (homozygous)	Spanish consanguinity	[237]
3. T412 → C	3-exon	Cys <sup>138</sup> → Arg	No detectable CRM in patient cells, COS expression low ProAct CRM no function, transport mutation; bacterial expression demonstrated the protein retained ganglioside transport function, but reduced interaction with Hex A	Acute (homozygous)	US Black	[34,154,155]
4. G506 → C	4-exon	Arg <sup>169</sup> → Pro	Premature degradation of the mutant GM2 activator, transport mutation	Acute		[238]
5. G160 → T	2-exon	Glu <sup>54</sup> → Stop	No detectable mRNA or CRM	Acute (homozygous)	Laotian, Hmong	[239]

that elements of the  $\beta$ -subunit (other than its active site [25]) are necessary to somehow correctly align the complex and allow hydrolysis of the ganglioside. Further functions that have been identified for the  $\beta$ -subunit are to greatly increase the stability of the resulting dimer and to facilitate the transport of the  $\alpha$ -subunit out of the ER (see below) (reviewed in [3,33,36]).

#### 4. The clinical and biochemical phenotypes of GM2 gangliosidosis

##### 4.1. Sandhoff and AB-variant versus Tay–Sachs disease

Unlike Tay–Sachs disease, no predominant ethnic group has been shown with a high Sandhoff disease (Table 5) or AB-variant (Table 6) disease incidence. To date only 8 patients with the AB-variant form have been reported [3,37] and five mutations characterized (Table 6). However, a number of geographic isolates, or demes, have been identified with a high incidence of Sandhoff disease. For example, 36 cases of acute Sandhoff disease, belonging to 27 families, most of which traced their ancestry to a geographically isolated area encompassing the northwestern region of the province of Cordoba in Argentina, were diagnosed over a 7-year period [38] (Table 5, row 6). In addition to the total deficiency of both Hex A and B activity in Sandhoff disease (compared with just a loss of Hex A activity in Tay–Sachs disease), other distinguishing clinical features of Sandhoff disease include the presence of organomegaly and occasional bony deformities, similar to those associated with infantile GM1 gangliosidosis. Clinical laboratory findings that may also distinguish the two disorders include occasional foamy histiocytes in the bone marrow as well as the presence of *N*-acetylglucosamine-containing oligosaccharides in the urine of patients with Sandhoff disease. Because of the few reports on AB-variant patients (Table 6), little can be generalized about their clinical phenotype. Most present with a disease similar to (including the cherry red spot), but perhaps slightly milder than the acute form of Tay–Sachs disease [37]. Biochemically, they have normal levels of Hex A and Hex B measured with both the MUG and MUGS artificial substrates.

##### 4.2. Other less-severe GM2 gangliosidosis phenotypes

Although the most severe infantile forms of GM2 gangliosidosis have a rather homogeneous clinical presentation (see above), there are less severe forms which show extreme variability in expression. Typically, the earlier the age of onset of clinical symptoms the more severe the disease. Thus, in the past, patients have been classified strictly according to their age at onset. However, because this system is so variable, i.e. dependent on the initial timing of the diagnosis, a more general nomenclature based on the different clinical phenotypes and recognizing the dominance of the encephalopathy has been suggested [3]: acute (the classical infantile form, see above); subacute (late infantile and juvenile forms); and chronic (adult and chronic forms).

Patients with subacute GM2 gangliosidosis present the same symptoms as acute patients; however, onset is delayed, with motor ataxia first becoming evident at 2–6 years of age, and death occurring by age 10–15 years. Chronic patients usually present with abnormalities of gait and posture between 2 and 5 years of age. Most of the patients with this condition are still living in their third to fourth decade of life. This is the most clinically variable type of GM2 gangliosidosis with extreme variability of symptoms and course which can even be found in the same family or sibship. Clinically, symptoms of spinocerebellar and lower motor neuron dysfunction are most prominent. Psychosis, usually of the schizophrenia type with slow personality disintegration, often with episodes of depression, is evident or develops in one-third of patients. Because of the diversity in clinical symptoms associated with the adult phenotype, patients have been previously misdiagnosed with an array of disorders, including spinal muscular atrophy, atypical Friedreich ataxia and Kugelberg–Weller disease [39–42].

##### 4.3. The critical threshold hypothesis

Whereas mutations that cause the complete loss of Hex A activity, e.g. partial *HEXA* or *HEXB* gene deletions (see below), give rise to the acute form of GM2 gangliosidosis which is a devastating disease (see above), it is believed that mutations leaving even very small levels of residual Hex A activity



give rise to diseases of later onset and milder course. Utilizing an assay system employing the GM2 activator protein and radiolabeled GM2 ganglioside as a substrate, a correlation between residual Hex A activity and the severity of the resulting disease has been determined [43]. Activities found for acute, sub-acute, and chronic patients were  $\leq 0.1$ , 0.5, and 2–4% of normal controls, respectively. Two clinically healthy probands with low Hex A activity were found to possess activities of 11 and 20% [44]. Thus, these data suggest a ‘critical threshold’, i.e. the minimum amount of Hex A activity required to keep the rate of GM2 hydrolyzed greater than or equal to the rate of ganglioside transport and incorporation into the lysosome, for Tay–Sachs and Sandhoff disease of between 5 and 10% of normal Hex A activity [45].

#### 4.4. Mouse models for the GM2 gangliosidoses

Mouse models have been made for Tay–Sachs disease, Sandhoff disease and the AB-variant. As in human, total Hex levels are  $\geq 50\%$  in the Tay–Sachs mouse (from Hex B), while levels in the Sandhoff mouse are  $< 1.5\%$  (from Hex S). Interestingly, whereas the Sandhoff mouse presents with a clinical phenotype similar to that observed in human patients, the Tay–Sachs mouse does not develop a clinical phenotype, despite having biochemical and histological evidence of GM2 storage. The storage material in this mouse also differed from human patients and suggested the reason for the lack of a phenotype. In the Tay–Sachs mouse, there was no elevation in GA2 (the asialo-derivative of GM2, Table 3) levels. In humans with Tay–Sachs disease the GA2 levels are  $\sim 10\%$  of the GM2 levels, with Sandhoff disease or the AB-variant form levels are 50% of GM2, Table 2 [4]. In the Sandhoff mouse, levels of GM2 were increased only  $\sim 3$ -fold. In humans with any of the three forms, 500-fold increases are seen (Table 2) [4]. However, in the Sandhoff mouse, there was a dramatic increase in GA2. Based on this observation and radioactive GM1 (Table 3) feeding experiments with mutant mouse cells, it was concluded that in mice, unlike humans, the conversion of GM2 to GA2 by a lysosomal sialidase is a functional alternative pathway. Because either Hex A or Hex B can hydrolyze GA2; although in vivo Hex

B hydrolysis is very slow [46,47], the normal levels of Hex B in the Tay–Sachs mouse are sufficient to dramatically slow down the storage of GM2 and GA2.

The Sandhoff mouse is fertile during the early part of its life, and was bred with the Tay–Sachs mouse to produce offspring with a total Hex deficiency. These mice display a severe clinical phenotype consistent with a combined gangliosidosis and mucopolysaccharidosis [48]. These data indicate that glycosaminoglycans like gangliosides are critical substrates for Hex. It would also follow from these data that the low residual level of Hex S in Sandhoff disease is sufficient to degrade MPS substrates in mice and likely humans, as initially suggested by Kresse et al. [26,49]. Because the hydrolysis rates for non-Activator assisted substrates, e.g. MUG and the MPSs, are  $\sim 4000$ -fold greater than for GM2 [25], it is likely the level of Hex activity necessary to prevent substrate accumulation (the critical threshold) is much lower for the MPSs than it is for GM2. Thus the 1.5% residual Hex activity from Hex S in the Sandhoff mouse is sufficient to prevent MPS-accumulation. Finally, normal bone marrow has been transplanted into Sandhoff mice. While the treated mutant mice still died of gangliosidosis, their life expectancy was doubled and there was evidence of a slower neurological progression of the disease [50]. This study supports the idea that bone marrow transplants will not be an effective therapy for lysosomal storage diseases with severe neurological involvement because of the difficulty in crossing the blood–brain barrier by the secreted enzyme.

The AB-variant mouse produced a clinical phenotype intermediate to that of the other two. Storage of GM2 was only slightly increased over the Tay–Sachs mouse. However, in this case some GA2 storage was also evident, but still at a level  $\sim 10$ -fold less than that of the Sandhoff mouse [51]. The lower level of GA2 storage suggests that this glycolipid can be hydrolyzed in the absence of the Activator, but that the Activator is likely required for an optimal rate of degradation.

Although earlier work had suggested the possibility that mouse Hex B could hydrolyze GM2 in the presence of Activator [52], a more recent study with the fully purified Hex isozymes from mice and recombinant mouse Activator, demonstrated that it can not [53]. This study went on to show that in

contrast to its human analog, the mouse Activator can effectively stimulate the hydrolysis of GA2 by mouse Hex A and as well, but to much lesser extent by mouse Hex B. Thus these mice models have demonstrable species-specific differences in the Activator, Hex A, Hex B, and a lysosomal sialidase, and indicate that making extrapolations from them to the human condition is risky.

### 5. Intracellular transport of the Hex isozymes and the activator

Like other lysosomal and secretory proteins, both the  $\alpha$ - and  $\beta$ -chains of Hex and the Activator contain cleavable signal peptides which cause their synthesis to occur on polysomes attached to the rough ER (Figs. 1 and 4). In the ER polypeptide chain folding, Asn-linked glycosylation, disulfide bond formation, and dimerization also occur. Asn-linked glycosylation is the first step in targeting the pro enzymes to the lysosome. The generation of a mannose-6-phosphate 'tag' on a high-mannose type oligosaccharide occurs (Fig. 2) by the sequential action of two enzymes, i.e. UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (in the *cis* Golgi network and *cis* Golgi) and *N*-acetylglucosamine-1-phosphodiester- $\alpha$ -*N*-acetylglucosaminidase, and requires that the monomer obtain its near native folding pattern [54]. There are three sites on the  $\alpha$ - and four on the  $\beta$ -pro-polypeptides that receive Asn-linked oligosaccharides in the ER, a fifth potential site near the C-terminus of the  $\beta$  is not glycosylated. Of these sites, the third on  $\alpha$ , and the first and fourth on  $\beta$  normally obtain the mannose-6-phosphate tag in the *cis* Golgi [11,55]. In keeping with the hypothesis that both subunits have very similar three dimensional structures, the phosphorylated third glycosylation site in the  $\alpha$ -chain aligns with the fourth in the  $\beta$ -chain (there is no potential glycosylation site in  $\alpha$  that aligns with the first site in  $\beta$ ) (Fig. 1). The phosphomannosyl residues serve as the essential components for the high affinity binding of lysosomal proteins to one of two receptors, MPR-CI (mannose-6-phosphate receptor, cation independent) and MPR-CD (mannose-6-phosphate receptor, cation dependent), in the *trans* Golgi network [56]. The protein-receptor complex is then transported to a late endo-

some where the lower pH induces the dissociation of the complex and the recycling of the receptor [57]. The generation of the mannose-6-phosphate tag is not 100% efficient, nor is the binding of properly tagged molecules to a MPR. If either one of these processes fail, the untagged and/or unbound pro-Hex molecule follows the cell's default pathway, i.e. it is secreted (Fig. 2). In the lysosome, the single chain pro- $\alpha$ - and - $\beta$ -polypeptides ( $\sim 68$  kDa each) undergo further proteolytic [13,58,59] as well as glycosidic [60] processing to produce the complex polypeptide structures, held together by disulfide bonds '[ ]', of the mature subunits (Fig. 1), i.e. [ $\alpha_p$  ( $\sim 14$  kDa)  $\alpha_m$  ( $\sim 56$  kDa)] [ $\beta_p$  ( $\sim 17$  kDa)  $\beta_b$  ( $\sim 24$  kDa)  $\beta_a$  ( $\sim 28$  kDa)] (reviewed in [3,14]). The conversion of the pro-polypeptides to the mature subunits is easily detected by Western blot analysis [61] and can be used as a marker for lysosomal incorporation [62].

The pro form of the Activator is also processed in the lysosome at its *N*-terminus to produce a mature chain of 162 residues. There is a single site for *N*-linked glycosylation at Asn<sup>63</sup>. As well the Activator has eight Cys residues forming four disulfide bonds which have recently been mapped to its primary sequence (Fig. 4) [63]. These bonds are likely responsible for the activator's great heat stability [34]. Depending on the composition of its oligosaccharide, the pro-polypeptide can have an  $M_r$  as determined by SDS-PAGE, of 22 kDa (high mannose type) or 24–27 kDa (complex type). As well, a small amount

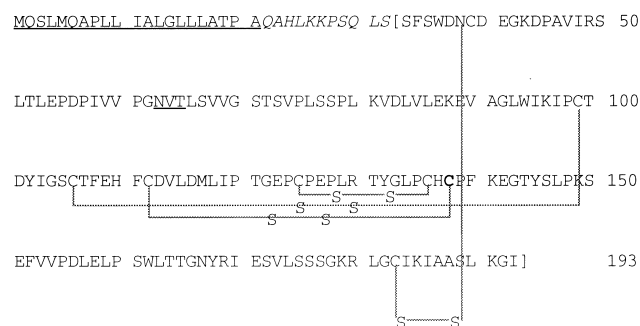


Fig. 4. Primary structure of the Activator protein. The proposed signal peptide is underlined, as is its single *N*-linked glycosylation site. The sequence of the mature lysosomal polypeptide is shown between '[ ]'. The four disulfide bonds present in the monomeric protein are also shown. C<sup>138</sup> is shown in bold. This residue has been linked to Hex A binding and when mutated to R results in the AB-variant form of GM2 gangliosidosis (Table 6).

of non-glycosylated Activator (20 kDa) is also made and transported out of the ER [64,65].

Two recent reports have studied the transport of the Activator in human fibroblasts [65] and human epidermal keratinocytes [64]. In the former report, the MPR pathway was identified as the likely major biosynthetic route for the incorporation of the Activator into the lysosomes. However, it was also demonstrated that a large percentage of the newly synthesized Activator did not contain the mannose-6-phosphate tag and was normally secreted. There is also a MPR-independent mechanism for the re-capture of the Activator from the extracellular medium [65,66] which is carbohydrate independent, but appears to require that the Activator be properly folded. This is likely the primary endocytotic route for the Activator; however, it is of a much lower affinity than re-capture through the MPR. In human epidermal keratinocytes only 10% of the newly synthesized Activator is phosphorylated, whereas 70% is retained intracellularly. Thus in these cells there is likely a major MPR-independent biosynthetic pathway used by the Activator to get into the lysosome. These data indicate that a large portion of newly synthesized Activator molecules are secreted by normal cells and can then be re-captured (with or without bound glycolipids) suggesting that the Activator may have other *in vivo* functions likely related to its alternate glycolipid transport function (Fig. 3).

## 6. Structure of the *HEXA*, *HEXB* and *GM2A* genes

The genes encoding the  $\alpha$ - and  $\beta$ -subunits have been isolated and characterized in detail. Both the *HEXA* and *HEXB* genes are split into 14 exons spanning  $\sim 35$  and  $\sim 40$  kb [23,67,68]. *HEXA* transcribes two mRNAs of 2.0 (major) and 2.3 (minor) kb both of which encode the same prepro- $\alpha$ -polypeptide [24]. *HEXB* transcribes a single 2.2 kb mRNA [58,69]. Exons 2–14 of the two genes have splice junctions at virtually identical positions in the coding sequence, i.e. within same aligned amino acid codon. The striking conservation of intron positions between the two genes provides a compelling argument that they arose by duplication of an ancestral gene. At the 3'-end, two polyadenylation sites are located in exon 14 of the *HEXA* gene (responsible for the two

mRNA species), while there is a single site in exon 14 of the *HEXB* gene. Primers and the PCR conditions needed to amplify the complete *HEXA* and *HEXB* exons and their flanking intronic sequences have been reported by Triggs-Raine et al. [70] and Wakamatsu et al. [71], respectively.

Unlike other sphingolipid activator proteins (SAP), i.e. SAP A–D, which are encoded by a single gene on chromosome 10 and synthesized as a large precursor polypeptide, prosaposin, the Activator is encoded by *GM2A* on chromosome 5 (reviewed in [33,35,37]). There is also a non-functional, processed pseudogene related to the *GM2A* gene on chromosome 3 [72]. *GM2A* is a small gene of at least 16 kb whose promoter has not been characterized. Three exons were identified and a fourth extrapolated to account for the remaining 81 bp of 5'-coding sequence not found in genomic clones. Among the three introns, only intron 3 has been fully sequenced, and the sequences of intron 1 and intron 2 and their exonic junctions remain to be determined [73]. *GM2A* mRNA is about 2.5 kb, but contains only 582 nucleotides of actual coding region. The majority of the remaining sequence consists of a long 3'-untranslated end [73–75]. We have recently completed the structure of the *GM2A* gene and developed primers and PCR conditions which will allow others to completely amplify the exons and their flanking intronic sequences [239].

## 7. Mutations in the *HEXA*, *HEXB* and *GM2A* genes associated with disease

### 7.1. Partial gene deletions

Three partial gene deletions have been described. One in the *HEXA* gene removes 7.6 kb from the 5'-end of the gene including exon 1 and is common in the French-Canadian population (Table 4, row 1) [76–78]. Two *HEXB* deletions also remove the 5'-end of the gene. The first is 16 kb long [18], spanning from 2 kb upstream of exon 1 to within the first 1.5 kb of intron 5 (Table 5, row 3) [79]. This mutation makes up a surprising 26% of all Sandhoff alleles [79]. Some confusion exists in the literature because a 50 kb *HEXB* deletion was also reported to be a high frequency mutation causing Sandhoff disease

Table 7  
5'-Splice junction

Exon				Intron						Consensus
-4	-3	-2	-1	+1	+2	+3	+4	+5	+6	
C <sub>29</sub> /A <sub>34</sub>	C <sub>38</sub> /A <sub>35</sub>	A <sub>62</sub>	G <sub>77</sub>	g <sub>100</sub>	t <sub>100</sub>	a <sub>60</sub>	a <sub>74</sub>	g <sub>84</sub> t <sub>50</sub>		
C	A	C	A	g	t	a	a	g → a	t	A-exon-4/IVS-4 <sup>b</sup>
T	C	T	G → A	g	t	a	a	c	c	A-exon-5/IVS-5 <sup>c</sup>
C	C	A	G → A	g	t	a	a	g	a	A-exon-7/IVS-7 <sup>d</sup>

<sup>a</sup>Percentage of wild-type splice junctions containing this nucleotide at the indicated position.

<sup>b</sup>The 'g<sup>+5</sup>a' mutation in the *HEXA* gene at the exon-4, IVS-4 junction (A-exon-4/IVS-5) results in a 3% steady-state level of mRNA (lacking exon 4) and infantile Tay-Sachs disease (Table 4, row 18).

<sup>c</sup>The G<sup>-1</sup> → A, i.e. G<sup>570</sup> → A, mutation results in no amino acid substitution, 3% of normal steady-state levels of mRNA (7% of this lacking exon 5), subacute Tay-Sachs disease (Table 4, row 32).

<sup>d</sup>The G<sup>-1</sup> → A, i.e. G<sup>805</sup> → A, mutation results in a Gly<sup>269</sup>Ser substitution, ~50% normally spliced mRNA, and the chronic form of Tay-Sachs disease (Table 4, row 49), primarily due to the amino acid substitution.

(Table 5, row 2) [80]. The size of this deletion was originally estimated by pulse field gel electrophoresis [81]. This size estimate turned out to be incorrect, and the 50 kb deletion was found to be identical to the 16 kb deletion [82]. The second *HEXB* deletion is indeed a 50 kb deletion, removing 25 kb 5' of the promoter up to intron 6 (Table 5, row 1) [83].

Because of their high frequency, the *HEXA* and 16 kb *HEXB* gene deletions have been well characterized [77,79]. The deletion mechanism for both *HEX* genes involved recombination between misaligned small homologous non-coding DNA sequences belong to the Alu family of interspersed repeated DNA. Patients homozygous for either of these *HEX* gene deletions have been described and present with the classical acute infantile form of GM2 gangliosidosis; indicating that this clinical phenotype results from the total lack of all Hex A activity. This conclusion also explains why the acute phenotype shows the least clinical variations (see above).

## 7.2. Mutations affecting mRNA splicing

The consensus sequences of the 5'- (Table 7) and 3'-splice sites (Table 8) are well established [84]. Of these nucleotides four are invariant. These include the first and last two nucleotides of each intron (IVS, intervening sequence), i.e. +1g and +2t (Table 7) and -2a and -1g (Table 8) [85]. Of the 13 mutations in the *HEXA* and *HEXB* genes that affect RNA splicing, seven involve one of these four nucleotides (Tables 2–4). Patients homozygous for these muta-

tions or heterozygous with a second null mutation, have no detectable mRNA and present with the acute phenotype [3]. Outside of mutations at these four nucleotides, the consensus sequences (Tables 7 and 8) have only a limited predictive usefulness. For example, two other *HEXA* gene splicing mutations are also associated with the acute phenotype, but would not have been predicted to totally preclude proper splicing based solely on the consensus sequences. The first is a 5-bp (tctcc) deletion, -8-12 IVS-9 (Table 4, row 65), which interrupts some of the string of 10 pyrimidine residues associated with the 3'-splice site, but whose overall importance in the splicing reaction has not been established (Table 8) [70]. The second is a 'g<sup>+5</sup>a' substitution in intron 4 (Table 4, row 18) [175]. A g<sup>+5</sup> is present in 84% of the characterized wild-type splice junctions, but an 'a<sup>+5</sup>' is found in another 9% (Table 7) [85].

The remainder of the splicing mutations allow some normal mRNA to be produced, which results in residual Hex A activity and a milder clinical phenotype. An interesting example of how the same substitution can have different outcomes depending on its context within the splice junction can be seen by comparing the G<sup>-1</sup>A substitutions in the last nucleotides of exons 5 and 7 of the *HEXA* gene (Table 4, rows 32 and 49). An A<sup>-1</sup> is found in about 8% of wild-type splice junctions, a G<sup>-1</sup> in 77% (Table 7) [85]. In the case of the exon 5 mutation, no amino acid substitution is encoded, but >97% of the mRNA produced is misspliced (producing low steady-state levels of mRNA). The mutation in

exon 7 resulted in a Gly<sup>269</sup>Ser substitution; however, steady-state mRNA levels are lowered only by 50%, the majority of which is properly spliced. While the abnormal splicing due to the exon 5 mutation is responsible for the homozygous patient's subacute phenotype [86], it is the amino acid substitution resulting from the exon 7 mutation (resulting in an unstable  $\alpha$ -subunit [87]) that is the major cause of patients' chronic Tay–Sachs phenotype [88]. The difference in the effects of these seemingly identical substitutions may result from the other components of each exon/intron junction. The exon 7 junction is a much closer match to the consensus than the exon 5 junction (Table 7). Therefore, exon 5 may have an inherently less stable splice site and the additional destabilizing effect of the substitution is enough to prevent the cell's splicing mechanism from recognizing it.

Three *HEXB* gene mutations affecting mRNA splicing are of interest. In all three cases, some properly spliced mRNA is generated resulting in detectable levels of Hex A ranging from 10% for asymptomatic individuals to 5% for two patients with subacute Sandhoff disease [89]. The distribution of

the major Hex isozymes in samples from individuals with either of these mutations is very similar, all have almost undetectable levels of Hex B. Thus the small amounts of pro- $\beta$ -chains being translated (with the wild-type sequence) are preferentially formed into the less stable Hex A heterodimers [89,90]. A mechanism to explain this unexpected observation is presented below.

The first of the above three *HEXB* mutations to be described was a 'g<sup>-26</sup>a' substitution at the 3'-end of intron 12 (Table 5, row 16). This substitution resulting in an mRNA encoding an extra eight amino acids between exons 12 and 13 (Table 8) [91]. Normal steady-state levels of  $\beta$ -mRNA are present with >95% encoding the additional eight amino acids. The second mutation is associated with patients whose biochemical phenotype was previously designated as 'Hexosaminidase Paris' because of their Hex A<sup>+</sup>/Hex B<sup>-</sup> isozyme pattern [44]. Despite the fact that these individuals are asymptomatic, i.e. their clinical phenotype is dramatically different from the individual with the first mutation, the correlations between their genotype and biochemical phenotype is nearly identical to that of the subacute patient's.

Table 8  
3'-Splice junction

Intron														Exon
-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1
y <sub>78</sub> <sup>a</sup> b	y <sub>81</sub>	y <sub>83</sub>	y <sub>89</sub>	y <sub>85</sub>	y <sub>82</sub>	y <sub>81</sub>	y <sub>86</sub>	y <sub>91</sub>	y <sub>87</sub>	n	c <sub>78</sub>	a <sub>100</sub>	g <sub>100</sub>	G <sub>55</sub>
c → a <sup>c</sup>	c → c	t → a	c → c	t → t	c → c	c → c	t	c	t	c	c	a	g	G
t <sup>d</sup>	g	t	a	a	c	g	t	t	a	a	t	a	g	C
t <sup>e</sup>	c	c	t	t	g	g	t	a	c	t	t	a	g	A
g <sup>f</sup>	a	t	t	t	a	a	a	t	t	t	t	a	g	G
t <sup>g</sup>	a	a	a	c	t	g	c	t	t	g	c	g <sup>-26</sup> → a <sup>-2</sup>	g	G

<sup>a</sup>Consensus (y = c or t; n = any).

<sup>b</sup>% of occurrences in wild-type splice junctions.

<sup>c</sup>*HEXA*-IVS-9: A  $\Delta$ t-c-t-c-c deletion results in a low steady-state level of mRNA lacking exon 10 and acute Tay–Sachs (Table 4, row 65).

<sup>d</sup>P1 = The wild-type sequence of IVS-10 and exon-11 in the *HEXB* gene. A 'C<sup>1252</sup>T' mutation at position +8 in exon-11 causes a Pro<sup>417</sup>Leu substitution in the protein that has been shown to cause no adverse effects; however, it also somehow results in a reduction in normal mRNA splicing, and promotes the use of a cryptic splice site in the middle of exon 11, P2<sup>e</sup>, or the skipping of exon-11 entirely, P3<sup>f</sup>. Both P2 and P3 contain premature Stop codons. This mutation results in variable clinical phenotypes, from very mildly affected to subacute forms of Sandhoff disease (Table 5, row 12).

<sup>e</sup>P2 = A cryptic splice site in the middle of exon-11 of the *HEXB* gene (footnote d).

<sup>f</sup>P3 = The wild-type sequence for IVS-12 in the *HEXB* gene (see footnote d).

<sup>g</sup>A 'g<sup>-26</sup>a' substitution in IVS-12 produces this alternative splice site with a<sup>-26</sup> moving to the -2 position, i.e. g<sup>-26</sup> a<sup>-2</sup>. The level of mRNA is normal; however, >95% is misspliced encoding the insertion of 8 amino acids. The <5% normally spliced mRNA is presumably responsible for the resulting subacute form of Sandhoff disease (Table 5, row 16).

The only difference is that cells from these individuals are able to produce residual Hex A levels about 2-fold higher than those of the subacute Sandhoff patient's ( $\sim 10\%$  of normal). These patients have an 18-bp duplication consisting of 16 bp of intron 13 and the first 2 bp of exon 14 (Table 5, row 20). This results in an in-frame insertion encoding an elongated pro- $\beta$ -chain (6 new amino acids) [89]. As with the other mutation, normal steady state levels of mRNA can be detected, with 90% of the mRNA encoding the additional residues. However, both mutations produce only small steady-state levels of elongated mutant pro- $\beta$ -chains, as detected immunologically ( $\beta$ -CRM). Additionally, the mutant pro- $\beta$ -chains are not phosphorylated and are recognized by antiserum specific for the unfolded form of the  $\beta$ -protein. These data indicate that the elongated  $\beta$ -polypeptides are not transported out of the ER [89]. Thus, the residual Hex activities in both cases arise from the 5–10% of  $\beta$ -mRNA that is properly spliced, i.e. normal  $\beta$ -subunits, not from the elongated mutant  $\beta$ -protein.

The third *HEXB* mutation, C<sup>1252</sup>T in exon 11, encodes a Pro<sup>417</sup>Leu substitution and is associated with a wide range of clinical phenotypes and is the common cause of chronic Sandhoff disease (Table 5, row 12). Since this nucleotide is 8 bp downstream from the intron 10/exon 11 junction, it was surprising to discover that its detrimental effect on residual Hex levels was not caused by the missense mutation, but was caused by a large decrease in normally spliced  $\beta$ -mRNA. The first description of the mutation was in a homozygous, 39-year-old Japanese patient with mental retardation and local panatropy [71]. The patient's steady-state  $\beta$ -mRNA level was reduced to 8% of normal. Three species of  $\beta$ -mRNA were characterized. P1 (the longest species) was the normal transcript. P2 and P3 were alternatively spliced mRNA (Table 8). The missplicing that generates either P2 or P3 produces a frame shift and a premature Stop codon. The ratio of these RNA species was P1:P2:P3; 11:2:1 [71]. Thus P2 and P3, like other mutant mRNA species that contain early Stop codons, are unstable (discussed below) and only  $\sim 6\%$  of normal mRNA is produced. This mutation was next identified in a large French-Canadian family. The subject was found to be heterozygous for the

C<sup>1252</sup>T in exon 11 and the 16-kb deletion mutation. The presence of this null allele would suggest that the patient should present with a more severe phenotype than the homozygous Japanese patient. Surprisingly, the patient was a 57-year-old man with normal intelligence, a 9-year history of severe watery diarrhea, and diffuse abdominal pain. His family also contained four other members (ages 51–61 years) who were apparently of the same genotype, but had no clinical phenotype. The same normal and misspliced mRNA species, P1, P2 and P3, were found in cells from this patient at about the same ratio [90]. Two other Italian patients have also been identified with the C<sup>1252</sup>T substitution. Both patients were heterozygous, the first with the 16-kb deletion, and the second with a mutation encoding a Cys<sup>292</sup>Tyr substitution (Table 5, rows 3 and 11). The clinical phenotype of the first patient at age 34 years was milder than the Japanese patient, but slightly more severe than the French-Canadian, presenting with normal intelligence and only an almost static lower motor neuron disorder. On the other hand, at 34 years, the second patient had, in addition, severe signs of cerebellar dysfunction, dysautonomia and peripheral polyneuropathy [92]. The reason for the different phenotypes associated with this mutation is unclear. It may involve small genetic variations between individuals in any one of the complex components of the mRNA-splicing machinery, or it may be related to variations in other genes directly associated with ganglioside biosynthesis and metabolism. Evidence from the study of 22 cystic fibrosis patients with splice-junction defects support the former conclusion. Even patients with the same CFTR mutations produced drastically different levels of normal transcripts in their respiratory epithelial cells [93].

The mechanism of how the above nucleotide substitution at an exon +8 position at a 3'-splice site can have such profound effects on mRNA splicing has yet to be determined. It has been suggested that exon sequences between +1 and +13 of 3'-junctions may be involved in binding the protein U5<sup>200</sup>, a part of the U5 small nuclear ribonucleoprotein particle (snRNP), and may play a role in aligning the 5'- and 3'-exons for ligation [94]. Clearly, there is more to be learned about mRNA splicing mechanisms.

### 7.3. Mutations producing premature Stop codons

Naively, one would predict that premature Stop codons would produce truncated proteins that may have some residual activity depending on how close to the normal C-terminus the new Stop occurs. This is not true for mutations in *HEX* and many other genes for two reasons. Firstly, early Stop codons often result in unstable mRNAs, for reasons yet to be determined. Secondly, proteins that are synthesized in the ER undergo a form of quality control by a resident ER system that recognizes abnormal proteins (even those that could be partially functional), and retains and degrades them (discussed in Section 7.4) [95].

Premature Stop codons can be generated directly through a nonsense point mutation or indirectly through a frame-shift. The latter can be generated through abnormal mRNA splicing (above), deletions, or insertions. There are numerous cases of these types of mutation causing GM2 gangliosidosis (Tables 4–6). In order to contrast the effect on steady state levels of mutant mRNA between inframe deletion/insertion mutations and those that generate frame shifts, first consider the example of the in-frame insertion resulting in ‘Hexosaminidase Paris’ given above. As well, an example of an in-frame deletion due to abnormal splicing is supplied by the major mutation among Japanese patients with infantile Tay–Sachs disease, a ‘g<sup>-1</sup>t’ substitution in intron 5 of the *HEXA* gene (Table 4, row 35). This mutation like the one producing Hexosaminidase Paris, results in normal steady-state levels of mutant mRNA [96]. However, in both cases, only low levels of mutant pro- $\beta$ - or pro- $\alpha$ -protein are detected (see Section 7).

The major Ashkenazi Jewish mutation is an example of a frame shift due to a small insertion producing a Stop at codon 431 (Table 4, row 74). Because of its high frequency, the effect of this mutation on mRNA production has been well studied. In patient cells there is no detectable steady-state mRNA; however, nuclear run off experiments confirm that the mutant *HEXA* gene is transcribed at a normal rate [97]. There have been eight reported nonsense mutations (Table 4, rows 6, 13, 30, 61, 67, 69 and 70; Table 5, row 10) none of which have been shown to result in detectable (Northern blot) levels of mu-

tant mRNA. Thus, premature Stop codons generally lead to unstable mRNA.

One exception to the above generalization is a single bp deletion mutation in exon 13 of the *HEXA* gene (Table 4, row 84). This mutation results in normal steady-state levels of  $\alpha$ -mRNA encoding a loss of 22 residues from the C-terminus of the pro- $\alpha$ -chain. However, only low levels of the mutant pro- $\alpha$ -chain can be detected [98]. These data suggest that the closer the Stop codon is to the 3'-end of the coding region the more stable may be the mutant mRNA. The correlation between the location of the premature Stop codon and the steady-state level of  $\beta$ -mRNA (normally codon 557) was probed in cells from three patients with acute Sandhoff disease [99]. Patients 1 and 2 were heterozygous for the common 16 kb deletion and either a substitution producing a Stop at codon 284 (Table 5, row 10), or a deletion producing a Stop at codon 451 (Table 5, row 14). The third patient was heterozygous for two deletion mutations producing Stops at codons 274 and 454 (Table 5, rows 9 and 13). The steady-state levels of  $\beta$ -mRNA in patient cells were quantitated for Stop codons at: 284, 0.8% (of normal  $\beta$ -mRNA); 451, 30%; and the combined 274 and 454, 1.8% [99]. Thus, because of the large disparity between the effects of premature Stop codons at 451 (30%) and 454 (<1.8%), there is only partial support for the idea that the further the Stop codon is 5' the more severe its effect on mRNA stability. Clearly more work is needed to elucidate the mechanism by which premature Stop codons produce unstable mRNA.

### 7.4. Missense mutations

To date, missense mutations at all but two codons in either *HEX* gene result in normal levels of mutant mRNA and a dramatic reduction in both mature  $\beta$ - and/or  $\alpha$ -protein (CRM) with a proportional reduction in Hex B and/or Hex A activity in patient cells. As well, in Tay–Sachs disease Hex B activity and  $\beta$ -CRM are elevated, whereas in Sandhoff disease levels of Hex S and  $\alpha$ -CRM are barely detectable, despite normal  $\alpha$ -mRNA levels [7,18,24,62]. These observations plus pulse-chase studies [62,100–102] suggest that mutant forms of either subunit, as well as the majority of wild-type  $\alpha$ -chain in cells deficient in  $\beta$ ,

undergo rapid degradation at an earlier stage in their biosynthesis and intracellular transport, i.e. the ER/*cis* Golgi network (reviewed in [36,103]) (Fig. 2).

Unlike the majority of cellular proteins which are synthesized in the cytosol, lysosomal, secretory, and plasma membrane proteins are synthesized in the oxidizing environment of the ER. For these proteins it is now well accepted that unless each folds into its near native conformation and in some cases forms a functional multimer with another subunit(s), it is unable to pass out of the ER/*cis* Golgi network and into the *cis* Golgi, where further Golgi transport and either secretion or plasma membrane insertion continues via bulk flow [104]. Thus, the difference in the secretion rates of various proteins is directly related to their rate of folding and if necessary oligomerization (reviewed in [105–107]).

The retention through recycling between the ER and the *cis* Golgi network of unassembled subunit(s) of multimeric proteins, even though properly folded [108], is achieved through interactions with resident proteins which are themselves normally recycled between these compartments [109,110], i.e. chaperones, such as GRP94; protein disulfide isomerase, PDI; BiP; Erp72; ER60; Calreticulin; and Calnexin (reviewed in [111]). These proteins are also believed to assist normal monomers in their folding, while retaining those that are deemed to be ‘misfolded’, e.g. because of mutation, and accelerate their degradation (Fig. 2) [112,113].

Other types of chaperones include ‘catalysts that regulate folding’, e.g. sugar processing enzymes, and ‘transport subunits and molecular escorts’, e.g. pro-peptides and specific subunits of oligomers [111]. In the case of the  $\beta$ -subunit of Hex it has been shown that at least partial Asn-linked glycosylation is required for efficient ER to Golgi transport [11]. As well, although the precursor forms of both Hex A and B have the same substrate specificity and kinetic properties as the mature forms (Hou et al. unpublished results), the *N*-terminal pro- $\beta$ -peptide (Fig. 1) is required for the proper folding of the pro- $\beta$ -polypeptide in the ER and transport to the Golgi [114]. Disease causing, missense transport mutations located in the  $\alpha_p$  or  $\beta_p$  sections of the subunits have also been described (Table 4, rows 5 and 6; Table 5, row 5).

It appears that for Hex dimer formation is neces-

sary for ER to Golgi transport and that unlike  $\beta$ -homodimers (Hex B), homodimers of the  $\alpha$ -subunits (Hex S) are not readily formed. Thus, the  $\beta$ -subunit can be viewed as a ‘transport subunit’ [111] necessary for transport of the  $\alpha$  in pro-Hex A out of the ER. Such a difference in subunit affinity would also allow the cell to specifically retain  $\alpha$ -subunits and increase their concentration in the ER/*cis* Golgi network, which in turn would promote the formation of heterodimeric Hex A by newly synthesized  $\beta$ -subunits (Fig. 2), rather than the more stable Hex B homodimers (reviewed in [36]).

The above transport mechanisms explain the lack of detectable Hex B, but not Hex A in cases where splicing mutations lower the levels of  $\beta$ -mRNA encoding normal pro- $\beta$ -polypeptides, e.g. ‘Hexosaminidase Paris’ (Table 5, row 20). They also explain why only low levels of CRM containing missense mutations, or small in-frame insertion or deletion mutations are found in patient samples. These mutations must either alter the monomers’ ability to initially fold or decrease the stability of its folded form which in turn reduces the concentration of folded monomers and inhibits dimer formation [87]. Alternatively, at the very least, the mutations must change some surface properties of the affected monomer sufficiently so that it is recognized as being ‘abnormal’ by one or more chaperones, resulting in its retention and degradation. Although it would seem apparent that degradation of these proteins would occur in the ER, two recent reports strongly suggest that at least in some cases, e.g. CFTR [115], degradation occurs in the cytosol through the ubiquitin–proteasome pathway [116]. If this hypothesis is correct, some unidentified retrograde transport pathway between the lumen of the ER and the cytosol must exist.

The presence of the above ‘quality control system’ in the ER suggests that subunits with missense mutations, even those associated with the most severe clinical phenotype, may not of necessity be incapable of forming a partially functional Hex A, but may be prevented from doing so by their increased affinity for one or more chaperones. In keeping with this hypothesis, when three  $\alpha$ cDNAs encoding different disease causing *HEXA* missense mutations (Table 4: row 46, Gly<sup>250</sup>Asp, subacute; row 49, Gly<sup>269</sup>Ser, chronic; row 79, Glu<sup>482</sup>Lys, acute) were individually over-expressed in COS-1 cells along with the wild-



type  $\beta$ cDNA, surprisingly high levels of Hex A activity were detected as compared to activity levels in patient cells [87]. Similar observations were made by other groups studying *HEXA* missense mutations associated with the chronic and pseudo-deficiency phenotypes (Table 4, rows 29, 43, 44, 46 and 49) [117,118], and by a group studying a Leu<sup>176</sup>Phe substitution in another lysosomal enzyme,  $\beta$ -glucuronidase, associated with mucopolysaccharidosis (MPS VII) [119]. The effect of over expression would be to greatly increase the concentration of Hex (or  $\beta$ -glucuronidase) pro-polypeptides (mutant or wild-type) in the ER and either help drive protein folding/dimer formation through a form of mass action [120–122], and/or more simply by saturating the chaperone(s) that recognize the mutant protein as ‘abnormal’. Thus, the major detrimental effect caused by many *HEX* missense mutations is at the level of intracellular transport rather than structure–function.

Because of the ER quality-control system the initial hope that we would learn a great deal about structure–function relationships within the two Hex subunits by characterizing missense mutations associated with GM2 gangliosidosis has not been fully realized. Early pulse-chase metabolic labeling experiments were only able to group these mutations into two classes. Those that were able to obtain their lysosomal targeting label (mannose-6-phosphate) and remain soluble, i.e. could be extracted from patient cells without detergent, and those that did neither. To obtain their targeting label, lysosomal proteins must be at least partially folded [54] and be able to enter the *cis* Golgi network [123]. It would follow that those mutant proteins that retain some ability to fold would also likely be associated with the less severe clinical phenotypes. Expression of such mutations in COS or even CHO cells (over expression is less pronounced in CHO cells) often produce reasonable levels of Hex which are characteristically also heat labile. Examples of such mutations are  $\alpha$ Gly<sup>269</sup>Ser [87]  $\alpha$ Tyr<sup>180</sup>His [117],  $\beta$ Pro<sup>504</sup>Ser [29],  $\beta$ Arg<sup>505</sup>Gln [124], and  $\beta$ Ala<sup>543</sup>Thr [125], all of which are associated with the chronic forms of GM2 gangliosidosis.

#### 7.4.1. Mutations that produce a ‘pseudo-deficiency’ of Hex A

Mutations at two codons in the *HEXA* gene are responsible for most of the false-positive results obtained by enzyme-based Tay–Sachs screening procedures where carrier status is defined as < 48% Hex A (of total serum Hex) [126,127]. It is fortunate from a historical perspective that these types of mutation are present at a very low frequency in the Jewish community. Otherwise they would have caused many unexplainable false-positive results in carrier-screening and more seriously in prenatal diagnoses. Furthermore, they would have led to even more confusion in the 1970–1980s over the genetic cause(s) of Tay–Sachs disease.

Two missense mutations resulting in an  $\alpha$ Arg<sup>247</sup>Trp or an Arg<sup>249</sup>Trp substitution in exon 7 produce a ‘pseudo-deficiency’ of Hex A (Table 4, rows 43 and 44). They have been reported to produce 0–15% of normal Hex A in serum, 13–24% in leukocytes and 8–26% in fibroblasts [118,128–130]. These mutations were initially reported to affect the  $\alpha$ -subunit’s ability to hydrolyze MUG and MUGS to a greater extent than its ability to hydrolyze the Activator:GM2 ganglioside complex (reviewed in [3]). However, Hex recognizes only the terminal  $\beta$ -linked GalNAc or GlcNAc residue of a substrate, making the nature of the aglycone moiety of little importance. Therefore, the artificial MUG and MUGS substrates represent the minimal structures needed for enzyme-recognition and  $\alpha$ -specificity, respectively, and while it has been shown to be possible for a mutation to affect the rate of Activator:ganglioside hydrolysis (a much more complex event involving protein–protein interactions) and not MUGS hydrolysis, e.g.  $\beta$ Pro<sup>504</sup>Ser (discussed below [29]), the reverse would be unlikely. Recently Cao et al. have shown that the lower activities associated with these mutations are, like other missense mutations proportional to the levels of mutant intracellular, mature Hex A protein, i.e. as determined by mature  $\alpha$ -CRM, present [118]. This report goes on to identify instability of the mutant  $\alpha$ -chain (as Hex A) either in the lysosome or in extracellular fluids as the cause of the apparent deficiency in patient samples. This is a novel biochemical phenotype, as all other characterized missense mutations that result in lower CRM, do so because of defects in ER-transport. Thus, it

can be concluded that the low level of steady-state lysosomal Hex A that is present, is fully functional and above the 'critical threshold', > 5% [45], necessary to prevent ganglioside storage.

Recent molecular modeling of human Hex using the crystal structure of bacterial chitinase [131] suggests that the residues involved in the pseudo-deficiencies may be located on the surface of the folded protein. As well, from a biochemical perspective, their change from charged hydrophilic to aromatic hydrophobic residues is highly non-conservative and would be expected to disrupt protein folding unless such residues were located on the surface of the protein [121]. The appearance of a hydrophobic residue on the protein's surface may serve as a recognition site for proteolytic cleavage in the endosome/lysosome, or alternatively as one of the signals for chaperone binding in the ER [132–135].

#### 7.4.2. The B1 variants of Tay–Sachs disease

The missense mutations at two codons have produced some clues to the active site of Hex. They are associated with an unique biochemical phenotype called the B1-variant of Tay–Sachs disease (Table 4, rows 26, 27, 28 and 48). A mutation at a third residue was also linked with this phenotype,  $\alpha$ Val<sup>192</sup>Leu (Table 4, row 36); however, it has recently been shown that this is a typical transport mutation [136].

Patients with the B1 variant were originally thought to have an activator defect (the AB-variant form of GM2 gangliosidosis), because they express both Hex A and B activities as assayed with neutral (common) substrates, e.g. 4-MUG. However, unlike the normal Hex A found in the true AB-variants, Kytzia et al. found that B1 variant Hex A was inactive toward an  $\alpha$ -specific, GlcNAc-6-sulfate containing substrate (as well as GM2 ganglioside even in the presence of added activator protein), and they suggested the presence of a mutation at or near the active site of the  $\alpha$ -subunit [137].

A hypothesis similar to that made by Kytzia et al. was advanced by Suzuki and colleagues [138,139] who identified the first specific mutation linked to the B1 phenotype in five of six patients examined, a G<sup>533</sup>A substitution encoding a change in  $\alpha$ Arg<sup>178</sup> to His (Table 4, row 27). Patients homozygous for this mutation present with a subacute phenotype,

whereas those that are heterozygous present with an acute associated second allele present with a noticeably more severe phenotype [140]. Suzuki and colleagues originally noted that Arg<sup>178</sup> is found in a stretch of eight identical amino acids in the aligned primary structures of the  $\alpha$ - and  $\beta$ -subunits and that the His substitution was predicted (by computer analysis) to cause a change in the secondary structure of the  $\alpha$ -polypeptide.

Two other mutations at the same B1-codon have been described (Table 4, rows 26 and 28),  $\alpha$ Arg<sup>178</sup>Cys [141] and  $\alpha$ Arg<sup>178</sup>Leu [70]. In these two cases, the patients presented with the more severe acute phenotype. This increased severity is consistent with biochemical data from the patients' samples which suggest that lower levels of mutant  $\alpha$ -chain were able to form heterodimers (Hex A) and exit the ER (detected as mature  $\alpha$ -CRM). In turn, these data correlate with the degree to which the amino acid substitutions are conservative, i.e. for Arg > His > Cys  $\geq$  Leu [142].

The knowledge of the exact  $\alpha$ -mutation, even when coupled with the biochemical characterization of the Hex A from B1 patients, did not fully define the biochemical impact of the Arg<sup>178</sup>His substitution on the  $\alpha$ -subunit, because of the presence of a normal, active  $\beta$ -subunit. It was not known whether the affected  $\alpha$ -subunit had undergone a partial change in substrate specificity, i.e. become  $\beta$ -like and could no longer efficiently bind negatively charged substrates, but could still hydrolyze neutral substrates, or if it had entirely lost its catalytic ability. Expression of a mutant  $\alpha$ -cDNA alone to produce a mutant form of Hex S for analyses could also be problematic due to this isozyme's over-sensitivity to any  $\alpha$ -amino acid substitution, e.g. as demonstrated by its failure to form  $\alpha$ Gly<sup>269</sup>Ser Hex S (associated with the mild chronic form of Tay–Sachs disease) [87,143]. To overcome these problems, we took advantage of the common evolutionary origins of the two subunits and analyzed the biochemical consequences of the B1-substitution in the  $\alpha$ -subunit by in vitro mutagenesis of the aligned codon in the  $\beta$ -subunit,  $\beta$ Arg<sup>211</sup>His, to produce a stable mutant homodimer. We found that the substitution did not affect dimer formation or cellular transport, but caused a near total loss of activity towards MUG. Consistent with the initial computer-predictions of its effects

on secondary structure (reviewed in [144]), we noted small additional changes in the lysosomal stability (corrected by growing cells in leupeptin) and the rate of processing of the pro- $\beta$ -subunit [61]. Although there was no re-activation of the mutant Hex B protein, these additional effects were totally eliminated when a more conservative substitution,  $\beta$ Arg<sup>211</sup>Lys, was made. Through kinetic studies and the use of Arg-specific modifying agents, we were able to conclude that  $\alpha$ Arg<sup>178</sup> and  $\beta$ Arg<sup>211</sup> are active site residues, likely part of the catalytic sites (normal  $K_m$ , but only 0.2% of normal  $V_{max}$  for MUG), in Hex [145]. However, the recent molecular modeling from bacterial chitobiase suggests that these active Arg residues are directly involved in substrate binding, interacting with OH3 and OH4 of a  $\beta$ -GlcNAc substrate, docking the substrate in its proper orientation in the active site [131]. These data are not inconsistent with ours if the very low level of immuno-precipitated Hex activity we were detecting was from the endogenous COS cell background, and we were not able to increase the MUG concentration sufficiently (because of its low solubility) to activate a mutant human Hex B with a highly increased  $K_m$ . We have recently developed a method for removing 99.99% of endogenous Hex from transfected cell media using an epitope-tag that does not interfere with ER-transport (Hou et al. unpublished data). A re-examination of Arg<sup>211</sup>Lys-Hex B demonstrated that it had a  $V_{max}$  of 0.2% of normal and a  $K_m$  increased more than 10-fold. The very low apparent  $V_{max}$  indicates the critical role this residue must play in catalysis. These data suggest that the Arg does bind the substrate, but likely binds much better to the transition state intermediate, thus assisting catalysis. Thus even if higher MUG concentrations were achievable in our initial studies, we would not have seen the increase in residual activity expected from a mutation that only effects  $K_m$ . More support for the bacterial model comes from the comparative molecular modeling of *Streptomyces plicatus* (Sp-Hex) [146]. The overall three-dimensional structure that was generated for Sp-Hex (also using the *Serratia marcescens* chitobiase X-ray structure [131,147]) was similar to the previously published model for human Hex [131]. For Sp-Hex, Arg<sup>162</sup> aligns with  $\alpha$ Arg<sup>178</sup>. When Arg<sup>162</sup> was converted to His and the enzyme purified from transformed *Escherichia coli*, it was found to

have a  $K_m$  increased by 40-fold and a  $V_{max}$  of 20% of wild-type Sp-Hex (for MUG  $K_m$  = 0.14 mM,  $V_{max}$  = 3.2 mmol h<sup>-1</sup> mg<sup>-1</sup>) [146].

We analyzed the second mutation associated with the B1 variant (Table 4, row 48),  $\alpha$ Asp<sup>258</sup>His [148], in the same manner as above. The two patients originally described were heterozygous for this mutation and  $\alpha$ Arg<sup>170</sup>Trp (associated with the acute phenotype, Table 4, row 24). They were found to have Hex A levels by MUG that were below that of Tay–Sachs carriers, but above that of patients. On the other hand, MUGS activities were at the level typical for an affected individual [149]. When we expressed a conservative substitution at the analogous  $\beta$ -residue,  $\beta$ Asp<sup>290</sup>Asn, we found that the mutant Hex B had a  $K_m$  for MUG 2.5-fold higher than the wild-type enzyme, and that the reduced apparent  $V_{max}$  we observed was primarily caused by a reduction in mature  $\beta$ -CRM, i.e.  $k_{cat}$  was estimated to be 70% of wild-type [150]. Thus, this mutation, and by extension the  $\alpha$ Asp<sup>258</sup>His appears to affect both substrate binding and intracellular transport. Data from the molecular modeling of human Hex based on bacterial chitobiase suggest that the  $\alpha$ Asp<sup>258</sup> is only indirectly involved in substrate binding (chitobiase Asp<sup>448</sup> forms a hydrogen bond with its substrate through a water molecule). Thus it plays a much less crucial role than  $\alpha$ Arg<sup>178</sup> and is not critically involved in stabilizing any transition state intermediate [131]. This conclusion is also supported by the molecular model generated for Sp-Hex (see above) [146]. In Sp-Hex Asp<sup>246</sup> aligns with  $\alpha$ Asp<sup>258</sup>. When Asp<sup>246</sup> was converted to Asn and the enzyme purified from transformed *E. coli*, its  $K_m$  was increased by 1.2-fold and its  $V_{max}$  was decreased by 2-fold [146].

The method of expressing an  $\alpha$ -mutation in the aligned residue in the  $\beta$ -chain is useful in differentiating between mutations that affect the active site and those that reduce intracellular transport. Active site mutations remain inactive, as described above, while transport or folding mutations give increased residual activities. Two such  $\alpha$ -folding mutations (Table 4, rows 29 and 49) have been analyzed by this method [87],  $\alpha$ Tyr<sup>180</sup>His, expressed as  $\beta$ Tyr<sup>213</sup>His [117], and  $\alpha$ Gly<sup>269</sup>Ser, expressed as  $\beta$ Gly<sup>301</sup>Ser. Both of these are associated with the chronic form of Tay–Sachs disease and result in an  $\alpha$ -chain that is unstable at 37°C, indirectly affecting

its ability to associate with the  $\beta$ . When either mutation was expressed as its  $\beta$ -analog, nearly normal levels of Hex B activity and protein were found as compared to COS cells transfected with the wild-type  $\beta$ -cDNA. However, in keeping with the conservation of structure–function relationships between the two subunits coupled with inherently greater heat stability of the  $\beta\beta$ -dimer, while both mutant Hex B enzymes were stable at 37°C, they had a decreased heat stability at 50°C.

#### 7.4.3. The $\beta$ Pro<sup>504</sup>Ser substitution

We found that two French-Canadian patients (sisters) with chronic Sandhoff disease were heterozygous for the common  $\Delta$ 16 kb *HEXB* allele [79,90] and a C<sup>1510</sup>T transition encoding a Pro<sup>504</sup>Ser substitution (Table 5, row 17) [29]. Since the former allele produces no  $\beta$ -mRNA, the latter is responsible for the 15–25% [90,151] residual Hex A activity (using MUG) we initially reported to be present in the patients' fibroblasts, and their chronic phenotype. Western blotting had also indicated a similar reduction in the amount of mature  $\beta$ -protein [90]. This level of Hex A activity is similar to that found in fibroblasts from pseudo-deficiency patients (8–26%, see above) and well above what is believed to be the critical threshold necessary to prevent GM2 storage. Thus it was difficult to understand why these two patients present with chronic GM2 gangliosidosis. Two possibilities were considered. First, that the  $\beta$ -mutation is somehow affecting the  $\alpha$ -active site, lowering its activity towards MUGS and GM2, e.g. a new type of B1-variant. Second, the mutant  $\beta$ -subunit is affecting the ability of Hex A to bind the Activator:GM2 complex. Using mutant and normal Hex A produced in co-transfected CHO cells, it was demonstrated that, like other *HEX* mutations associated with chronic phenotypes (see above) the mutant  $\alpha\beta$ Pro<sup>504</sup>Ser Hex A had decreased heat stability coupled with an increased retention in the ER. However, the  $K_m$  values of the mutant Hex A for both the MUG and MUGS substrates were normal. Finally we assessed the ability of the mutant Hex A to hydrolyze its natural substrate, the Activator:GM2 complex. These data demonstrate that the  $\beta$ Pro<sup>504</sup>Ser mutation reduces the ability of the mutant Hex A to hydrolyze GM2 in the presence of human Activator by 3-fold. When this reduction in the specific activity

of the mutant towards GM2, but not MUG or MUGS, is factored into our residual Hex A activity measurement, the patients' activity levels are reduced to 3–9% of normal. This is very close to the critical threshold values discussed above and is consistent with the chronic phenotype observed in the patients.

Recently we [152] and others [153] have reported the characterization of  $\alpha$ - $\beta$ -fusion proteins. Although some of our conclusions differed, both studies concluded that the C-terminus of the  $\beta$ -polypeptide is important for the correct binding of the Activator:GM2 complex. The characterization of this novel, naturally occurring mutation strengthens these conclusions and identifies the region surrounding Pro<sup>504</sup> as the area in the C-terminus most likely to be responsible for this function.

#### 7.4.4. The Cys<sup>138</sup>Arg substitution in the Activator

There have been only four mutations described that are associated with the rare AB-variant form of GM2 gangliosidosis (Table 6, row 3). Of these the Cys<sup>138</sup>Arg was the first to be described [154,155] and is the most interesting from a structure–function point of view [34]. Like other missense mutations in the Hex A subunits, the Cys<sup>138</sup>Arg substitution results in the mutant protein being retained and degraded in the endoplasmic reticulum of the patient's fibroblasts [155]. In order to characterize the biochemical effects of this substitution, we expressed the mutant protein in transformed bacteria<sup>1</sup>. The mutant protein retained 1–2% of the wild-type's specific Hex A co-factor activity. The presence of even this small amount of activity in the mutant protein coupled with a nearly normal CD spectrum, strongly suggest that no major tertiary or secondary structural changes, respectively, had occurred due to the mutation. However, we found that the mutant protein had a 14-fold reduction in its heat stability at 60°C, suggesting some localized change in tertiary structure. The loss of a disulfide loop was chemically confirmed, which probably is responsible for this decrease. A kinetic analysis detected a large increase in the apparent  $K_m$  of Hex A for the mutant Activator;

<sup>1</sup> Bacterial expression requires refolding of the Activator from inclusion bodies [32,66,156]. Similar refolding methods do not work for the Hex B (unpublished data).

however, there was no apparent change in  $V_{\max}$ . A fluorescence dequenching assay was used to evaluate the ability of the mutant protein to transport lipids and bind GM2 [156]. These assays detected no difference between the wild-type and mutant proteins, indicating that the Cys<sup>138</sup>Arg substitution has no effect on these functions. We conclude that the mutation specifically affects a domain in the Activator protein that is responsible for the recognition of the Activator:GM2 complex by Hex A. Recently the four disulfide bonds of the Activator have been mapped by Schütte et al. (Fig. 4) [63]. Their study adds support to our hypothesis and localized the domain to the central portion of the Activator. They note that the bond between the first and last Cys (39–183) is a feature that is shared with other sphingolipid activator proteins (needed for lipid binding?), SAP B and C, which are formed post-translationally from prosaposin (reviewed in [37]). The other three disulfides occur within the middle third of the Activator monomer in a stretch of 39 residues, 125–136, 99–106 and 112–138, with the latter forming a ‘clamp’ around the former (Fig. 4). It was further noted that seven of the protein’s 17 Pro groups also reside in this region. These data suggest that the central part of the Activator is kept in a restricted conformation, likely needed for stability and to fulfil some specific function, e.g. interacting with Hex A [34].

## 8. Summary

The elucidation of naturally occurring mutations associated with each of the three GM2 gangliosidoses in either the *HEXA* (encoding the  $\alpha$ -subunit of Hex A, Tay–Sachs), *HEXB* (encoding the  $\beta$ -subunit of Hex A, Sandhoff), or *GM2A* (encoding the GM2 activator protein, AB-variant form) genes has made some genotype–phenotype (biochemical and clinical) correlations possible (Tables 4–6). The characterization of the effects of a few of these mutations on the translated protein have led to a better understanding of normal cell biology and given clues to the location of functional residues and/or domains in Hex A and the Activator. The effects of many others on the protein are linked with the ‘quality control’ system in the ER and have focused attention on the need to understand the mechanisms involved in the identi-

cation, retention and degradation of ‘abnormal’ and/or unassembled proteins in this organelle. More general questions have been posed by the biological consequences of other *HEXA* and *HEXB* mutations affecting mRNA processing, transport and/or stability. The history of the study of Tay–Sachs disease is an excellent example of how the interest in a human disease can stimulate research that has resulted in advances in a number of more general biological and biochemical fields.

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